# MEASUREMENT, PRODUCTION AND PRESERVATION OF THE HEMOLYTIC ACTIVITY OF GUINEA PIG COMPLEMENT

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# I

## INTRODUCTION

Guinea pig serum is the most important source of complement for the Wassermann and other complement fixation reactions. Although numerous reports have appeared regarding some of the factors which affect the hemolytic activity of complement, there is still considerable disagreement concerning the influence of certain factors. It seemed desirable, therefore, to investigate several of the more important conditions influencing the production of a satisfactory complement. Among those concerning the guinea pig itself are age, weight, sex, litter mate differences and the effect of feeding, sunlight, and temperature.

Factors involved in obtaining and preparing sera, such as pooling the blood of several animals and starving the animals before bleeding, were also measured. In addition, it seemed advisable to measure the influence of the diluting fluid upon hemolytic titer, and the possibility of correlating hemolytic titers and surface tension measurements.

After investigation of those factors that may influence the measurement and production of a hemolytically active complement, it would seem essential to preserve this activity for use in the complement fixation reaction. Since there is a wide divergence of opinion regarding suitable methods for the preservation of complement, comparisons were made of the procedures proposed and modifications these suggested.

# II

## HISTORICAL

Physiological Saline: Various suggestions have been made regarding the possible effects of saline upon the hemolytic activity of guinea pig complement. In a study of the precise titration of complement, Brooks (7) used a solution similar to Ringer-Tyrode's solution containing 8 gms. of sodium chloride, 0.2 gm. of potassium chloride, 0.2 gm. of calcium chloride, 1 gm. of sodium bicarbonate, and 0.05 gm. of monobasic sodium phosphate, made up in a liter of distilled water. This physiologically balanced solution was used in place of physiological saline as a diluent and aided in giving more accurate titrations.

The hydrogen ion concentration seemed very important to Mason and Sanford (34) and they urged the use in serology of a constant physiological salt solution. Their buffered solution contained 7 gms. of sodium chloride, 1.7 gms. of secondary sodium phosphate, and 0.2 gm. of primary potassium phosphate to each liter of double distilled water, with a pH of 7.4 to 7.8. They stated that the optimum hydrogen ion concentration for amboceptor titration was between pH 7.0 and 8.4. Boiling and the addition of carbon dioxide changed the hydrogen ion concentration in distilled water easily, while in tap water it did not change so greatly because of the natural buffers present.

The work of Rockwood (48) indicated that the effective range of acidity for biological hemolysis was between a pH of 6.0 and 8.3 with a broad optimum near neutrality. Acid would partly destroy both the amboceptor and complement. Mason and Sanford's buffered salt solution, although stabilizing a variable factor in the Wassermann test, generally

was not necessary, since, except in extreme cases, the buffering power of the cells was usually adequate.

Kellogg and Wells (26) did not consider the reaction of saline solutions of any great importance. Salines prepared with tap and distilled water of the same pH gave a low titration with distilled water and a very high one with tap water. They considered that the high titer was due to the presence of magnesium in the tap water. By adding 0.1 gm. of magnesium chloride or sulphate to a liter of physiological saline solution, the specific lysis of red cells was increased, and higher complement and amboceptor titers were obtained.

Kolmer (27) stated that sterilization of saline in an Arnold for an hour (for 1,000 cc., or a shorter time for a smaller bulk) was preferred to autoclaving because of the concentration of salt in the latter process due to the additional loss of water. Kolmer (28) also recommended the use of 8.5 gms. of chemically pure sodium chloride as the optimum amount of salt. He mentioned that the pH of the saline was of practical importance but not the sole factor to be considered in the selection of a good saline. According to Kolmer, the buffered solution of Mason and Sanford was somewhat hemolytic when made up in distilled water and was not so satisfactory as when prepared with Philadelphia tap water, although the range of pH for both was 7.3 to 7.4. He was of the opinion that "the natural buffers of tap water are likely to be more satisfactory in relation to serum hemolysis than artificially buffered distilled water." If saline prepared of distilled or tap water should be unsatisfactory, Kolmer recommended that 0.1 gm. of magnesium sulphate or chloride be added to each liter in accordance with the suggestion of Kellogg and Wells (26).

Most recent books on laboratory methods recommend distilled water for physiological saline. Wadsworth (62) suggested that physiological saline containing 0.85 per cent sodium chloride should be used for preparing suspensions of red cells, and for making all dilutions. The required quantity of distilled water was to be added to the salt, dissolved, filtered through paper, dispensed, and sterilized at 121° C. for twenty or thirty minutes, depending on the bulk. It was found unnecessary to compensate for small losses of water during sterilization, as the resulting salt concentration was about 0.88 per cent. According to Kolmer and Boerner (31), one liter of tap or distilled water should be added to 8.5 gms. of dry, chemically pure sodium chloride, filtered through paper into a flask fitted with a gauze-covered cotton stopper, and sterilized in an Arnold for an hour.

Gradwohl (21) recommended the use of 8.5 gms. of sodium chloride per 1,000 cc. of distilled water. This solution was filtered through paper and 500-cc. brown bottles were filled about two-thirds full, plugged with non-absorbent cotton, which was then covered with paper, and the bottles then sterilized in the autoclave for thirty minutes at fifteen pounds' pressure. He stated that fresh saline should be made once or twice a week, and, if not stored in brown bottles, should be placed in a dark cabinet.

Wadsworth, Harris and Gilbert (64) stated only that "the diluent for all reagents should be a salt solution containing chemically pure components isotonic for red cells."

Surface Tension Measurements: Explanations of immunity reactions are frequently dependent upon the colloidal nature of the sera. It was thought that the surface tension of the blood sera might possibly be correlated with the hemolytic titer.

Blood serum, which is colloidal in nature, loses its hemolytic activity when heated to 56° C. It has been pointed out by Zinsser (66) that during heating there is an aggregation of particles which causes a lowering of surface tension. Upon standing, the complement spontaneously reactivated itself and was accompanied by a gradual restoration in surface tension. It was believed that the gradual deterioration of complement on standing might be due to slow settling out of colloidal suspensions. Shaking has been reported to cause inactivation of complement, probably by coagulation. Whether this type of inactivation is accompanied by a drop in surface tension has not been investigated.

Hans Schmidt (53) reported that the surface tension of a serum did not permit any inference to be drawn as to its complement activity. Segale (54) verified the statement that heating serum to 56° C., or aging until complement activity had disappeared, caused an alteration in surface tension. Ultra-microscopic examination revealed aggregates of particles and the removal of such particles restored the surface tension to its original value. The addition of such particles to fresh serum caused a lowering of surface tension but did not interfere with the complement activity. Therefore, he concluded that particles formed in old or heated sera which lower surface tension have nothing to do with complement activity. Both Schmidt and Segale recorded their work before the advent of the du Noüy tensiometer.

Brooks (6) measured surface tension with a Traube stalagmometer and found that that of a 5 per cent complement solution decreased slightly after inactivation by ultra-violet light. Though this change of surface tension occurred, it was probably more like that found after thermoinactivation and was not the cause of the change in hemolytic power.

The most complete work on the surface tension of serum has been done by du Noüy (39). Through immunization experiments, du Noüy (40, 41) showed that immune animals demonstrated a greater time drop than normal animals. The time drop is the difference between the initial or dynamic surface tension and the static surface tension measured after a two-hour period when all the surface active substances are adsorbed in the surface layer. The maximum time drop was usually found in dilutions of 1:10,000. The time drop of the sera of immunized animals reached a maximum in about thirteen days; then decreased and finally disappeared in about thirty days. Further immunization of the same or different kind caused no more change. The injection of an antigen resulted in a physicochemical disturbance that could be followed by a decrease in the static value of the surface tension of the diluted serum. It was possible, according to du Noüy, that this reaction was independent of antibody formation.

Yagle (65), following the idea that antibody production increased the time drop of serum, could find little value in surface tension measurements of normal and syphilitic sera. She concluded that the reagins were not more surface active than other substances found in normal sera.

Ramsdell (44) used du Noüy's tensiometer for making the surface tension measurements to obtain evidence of a denaturing effect of the precipitin reaction upon either the antigen or the immune serum, and obtained negative results. Using the serum from sensitized guinea pigs, Ramsdell (43) showed an increased time drop over that of normal animals for about the same period of time as du Noüy (40, 41), and concluded that antibody formation could not be involved since the sensitized state remained after the surface tension of the serum had returned to normal.

Following the intravenous injection of gum shellac, Hayman (23) was able to detect an increase in the time drop of the diluted serum. He believed that this indicated changes in the physicochemical state of the plasma.

Production of Complement: Conflicting reports in the literature on the factors to be considered in producing a suitable complement for the complement fixation reaction together with personal observations on complement deficiency made it desirable to study some of these conditions.

Rich (47) found a strain of complement-deficient guinea pigs at the Vermont Agricultural Experiment Station. Genetic studies indicated that the complement was not a universal blood factor and could be controlled by selective mating in conformity with Mendel's law. Hyde (25), after ten years' work with the complement deficient strain of guinea pigs from the Vermont Experiment Station, believed that this lack of complement was a character which was inherited as a simple recessive Mendelian unit. Ruediger (51) was able to improve his collection of several hundred guinea pigs over a period of three years by breeding only those with good complement.

The work of Friedberger and Gurwitz (15) indicated that while sera from guinea pigs weighing from 150 to 600 gms. showed marked differences in complement content, they were wholly independent of age and weight. Kolmer (29) did not find any variation in hemolytic activity between a group of pigs weighing from 200 to 280 gms. and another group weighing between 400 and 580 gms., but did recommend the heavier animals for use, as the sera of the younger ones were more likely to be sensitive to the anticomplementary activity of antigen and serum. As a source of complement, Cummer (10) recommended pigs weighing 400 gms. or more because it

was poor economy to use small pigs yielding little blood and that weak in complement.

Browning (8) stated that the complement content of guinea pigs tended to be lowest immediately after birth but underwent a marked increase during the first three months of life. Friedberger and Gurwitz (14) found that the complement titer of new-born guinea pigs was about the same as that of the mother. Hyde (25) found no appreciable difference between the complement titer of young guinea pigs and that of mature animals whereas Osborn (42) stated that there is a marked tendency toward low complement titer in the blood of young rats.

Nutrition might conceivably have an effect on the production of complement. Simola and Brunius (57) reported that neither A or C avitaminosis nor the administration of vitamin D in the form of irradiated ergosterol had any effect on complement formation in guinea pigs. Working with rats on a diet deficient in vitamin A, Osborn (42) concluded that the blood complement content was lower than that of controls receiving cod-liver oil. While not bearing directly on the subject of complement, Madsen, McCay, and Maynard (33) found it difficult to raise guinea pigs using a synthetic diet free from hay and grain but including cod-liver oil or vitamin A-D concentrate. Cod-liver oil added to the diet caused muscle degeneration and other pathological conditions sooner than vitamin A-D concentrate. No injury occurred when the animals were on a natural diet whereas natural foods plus cod-liver oil produced muscle injury. The muscle degeneration appeared to be caused by a synthetic diet and also by cod-liver oil, either one alone being effective and both together acting more rapidly. The report pointed out the uselessness of feeding cod-liver oil, vitamin A-D concentrate, or synthetic diets to

guinea pigs. Hilgers and Zain (24) stated that acid and alkali diets generally had no effect on complement titer.

A report of the United States Department of Agriculture (59) recommended that guinea pigs be given water, salt, and dry grains such as oats, bran, or chopped grain. In addition, the animals should have hay and a daily feed of such green stuffs as cabbage, lettuce, celery tops, spinach, or kale. Rich (47) could find little evidence that diet had any effect on complement titer, using many hundreds of guinea pigs and feeding a variety of foods over a period of years. Kolmer (29), although admitting the lack of systematized and scientifically regulated diet experiments, stated that a mixed diet which maintained the animals in good health and normal weight was all that was necessary to obtain acceptable complement. In order to prevent dilution of blood by hydremia, Cummer (10) recommended that the pigs be put on a diet of oats only for at least a day before they were bled. Austin (2), working on the non-specific fixability of guinea pig serum with Bordet's antigen, kept the animals on a diet of cabbage, alfalfa and oats. The alfalfa was fed dry in winter and green in summer. Browning (8) stated, based on the work of Griffith and Scott, that in guinea pigs the state of nutrition had no definite relation to the complement content of the serum. Ruediger (51) found little difference in the titer of guinea pigs fed during the winter on various mixtures of such vegetables as potatoes, lettuce, carrots, cabbage, and apple peelings, in addition to a constant feed of bread, hay, and oats.

Kolmer (29) recommended the use of large, well-nourished animals, males preferred, which had not been fed within twelve to twenty-four hours of the time of bleeding, for securing satisfactory complement. He also

avoided using pregnant animals despite the fact that he found little difference between the titer of pregnant females and male pigs of the same weight, and suggested that the pooled sera of three or more pigs should be used. Cummer (10) stated that it is advisable to avoid pregnant animals and to use the pooled sera of three or more pigs in order to obtain satisfactory complement, since there is a considerable variation between different pigs. Wadsworth (63) made the statement that three, but preferably six or more pooled sera are desirable, and to avoid chylous serum advised not bleeding until several hours after feeding. Simmons (56) suggested that blood for complement be obtained from three or more full-grown, healthy male guinea pigs. Although giving no reason, he stated that females should be avoided because of the possibility of pregnancy. A deficiency in complement was associated with pregnancy according to Browning (8). On the other hand, Rich (47) found that neither castration nor pregnancy affected the complement content of guinea pigs. Gradwohl (20) made no recommendations regarding the guinea pigs used as a source of complement, but merely stated that complement varied from time to time in animals and that it was present in particularly large amounts in the blood of guinea pigs. Wadsworth, Harris, and Gilbert (64) stated that healthy animals, preferably males, should be tested for hemolytic activity and fixability, and the pooled sera of three or more satisfactory animals should be used. The same statement also appeared in a report by Gilbert (16).

Little information has been available on the effect of sunlight and temperature upon guinea pigs. A temperature below 65° F. was unsuitable for guinea pig management according to a report of the United States Department of Agriculture (59). Remlinger and Bailly (45) found that if rodents were left in the sun for as short a period as half an hour they

would be found dead and concluded that the susceptibility of rodents is probably due to their habitual sheltered existence.

Preservation: Numerous procedures for preservation have been reported. Shirvindt and Liberman (55) advocated for storage at room temperature the addition to fresh complement of 10 per cent crystalline sodium chlorate with 4 per cent crystalline boric acid, and for storage at $5^{0}$ C. the addition of 10 per cent crystalline sodium acetate with 4 per cent crystalline boric acid. Both preserved complement equally well. According to Sonnenschein (58) complement was better preserved by using 10 per cent sodium acetate with 4 per cent boric acid than by 10 per cent sodium chloride with 4 per cent boric acid.

Mirdamadi and Giese (35) reported that by mixing equal amounts of 12 per cent sodium acetate containing 4 per cent boric acid and fresh guinea pig serum, four complements maintained titers equal to that of fresh serum for 114, 66, 22, and 32 days respectively. Austin (1) found that a 25 per cent salt solution would preserve complement for from two to three weeks when stored in an ordinary test tube at ice box temperature.

According to the work of Ginsburg and Kalinin (18) full complement activity was retained for three to four months at room temperature when 10 gms. of sodium chloride with 4 gms. of boric acid were added to 100 cc. of guinea pig complement, using these in dry form rather than adding them in solution as most other investigators had done. The same workers (17) reported the use of 5 per cent strontium chloride with 4 per cent boric acid. Ruffner (52) believed that 10 per cent sodium acetate or sodium chloride with 4 per cent boric acid

were the best of many preservative substances studied since the complement so treated kept four weeks with little or no change.

Complement diluted to 40 per cent with a 12 per cent sodium acetate solution made up in physiological saline retained its activity for three months at ice box temperature, and for several weeks at room temperature, according to Rhamy (46). He pointed out that the acetate was not "antibacterial," since growth would take place when the serum was contaminated, but that it preserved complement in some way by a physicochemical action which involved entering into a loose combination with the complement.

Ronchése (49) added 0.04 gm. of sodium fluoride per cc. of complement and found that its hemolytic activity remained constant during the first five days; it decreased "two-fold" during the next five days, and "three-fold" during the third five days.

Kolmer (32) investigated sixteen methods for the conservation of complement. He concluded that sodium chloride (0.17 gm. for each cc. of complement) and storage at a low temperature yielded the best results. He recommended the methods of Thompson and Neill, who added 0.1 cc. of a saturated sodium chloride solution to each cc. of fresh complement, when dilutions of 1:10 were to be used, and reported satisfactory preservation for two weeks when the treated complement was kept at a low temperature. Kolmer (30) advocated the use of 25 per cent sodium chloride when a complement dilution of 1:30 was to be used. In a report of the National Institute of Health (36) on the technic of the Wassermann test, complement was preserved by the addition of 0.1 cc. of a saturated sodium chloride solution to each cc. of serum, resulting in the maintenance of a satisfactory titer over a period of six weeks.

Complement preserved with 17 per cent sodium chloride with 0.25 per cent chinosol to maintain sterility kept the activity of the complement at its original level for twenty days according to Glover (19).

Using various gases such as hydrogen, nitrogen, carbon monoxide, and carbon dioxide to displace air, Valley and McAlpine (61) found that only carbon dioxide was valuable in the preservation of complement. Under experimental conditions, complement titer remained unchanged in an atmosphere of carbon dioxide for several months. Valley (60) explained the preservative action of carbon dioxide as due to the creation of conditions that favored reduction and prevented oxidation.

Ruediger (50, 51) used glycerol and freezing as methods for preserving complement. He reported that Rhamy's method of preserving complement by sodium acetate was a failure in his hands. Norton, Barfield, and Falk (37) found that at 37° C. complement deteriorated in about three days; at 4 - 6° C. it maintained its original titer for forty-eight hours, and at the end of seven days the titer had decreased one-half to one-third that of the original. When frozen and held at -10° C. it was usable for two weeks. Complement kept at -12° C. retained its hemolytic power for six weeks according to Browning and Mackie (9). Bigger (3) determined the loss of complement activity of guinea pig serum at 50°, 40°, 30°, 20°, 9°, and 1.5° C. and found more rapid losses occurred as the temperature increased. About 75 per cent of the complement was lost in from six to seven days when stored at 9° C. At 1.5° C. the same loss occurred over an average period of thirty-eight days.

Dean (11) failed to preserve complement titer in a dried form after precipitation with alcohol and ether.

Flosdorf and Mudd (13) reviewed the development of drying as a method for the preservation of complement and described a procedure and apparatus for the preservation of sera, miscellaneous proteins, enzymes, viruses, and bacteria in "lyophile" form. The method involved freezing at low temperatures, followed by a rapid dehydration under high vacuum while in the frozen state. The process was continuous and was carried out in the containers in which the material was finally sealed under the original vacuum. The containers could then be stored and distributed as needed. The complement was readily restored to its original state by the addition of distilled water and the restored complement then used exactly as fresh complement. Complement preserved by this method was best stored at a temperature of 2 - 6° C. Storage for at least ten months by this method did not result in any detectable drop in titer.

Eagle, Strauss, and Steiner (12) found that complement which had been dehydrated from the frozen state using the Flosdorf-Mudd apparatus retained its full hemolytic activity throughout the duration of the experiment (eight months) when stored at 2 - 6° C. The titer was indistinguishable from that of freshly bled and salted complement which was used within three or four days. A greater uniformity in complement was obtained by Boerner and Lukens (4) who used "lyophile" complement prepared from the pooled sera of from ten to fifty guinea pigs. There was very little waste and its use obviated the necessity of obtaining complement during the summer. These same workers later (5) reported that "lyophile" complement stored at 8 - 10° C. retained its full hemolytic power for twelve months. After a year the dehydrated complement began to show some deterioration.

Greaves and Adair (22) recently described a procedure for the desiccation of antisera, complement, and bacterial cultures. The substances to be dried were frozen by rapid evaporation in a high vacuum in the presence of phosphorus pentoxide. Pooled guinea pig complement preserved by this process over a four-month period maintained a titer comparable to that of pooled fresh complement.

# III

# EXPERIMENTAL

## A. Measurements

Before investigations were carried out on the production or preservation of complement, it seemed desirable to make certain preliminary measurements, both in order to standardize the succeeding technique and to ascertain the effect on complement titer of certain factors, including sex of animals, pooling of blood, and removal of feed before bleeding.

### 1. Complement Titers of Guinea Pigs With and Without Feed for Eighteen to Twenty-Four Hours Before Bleeding.

Preparation of Materials: In obtaining the blood for complement, the guinea pigs were lightly etherized and held by an assistant. The point of maximum cardiac pulsation was selected, alcohol was used to sterilize the skin, and the proper needle, fitted to a B-D Medical Center syringe, was inserted at this point and 2 cc. of blood withdrawn. The blood was placed in a sterile agglutination tube and slanted. The syringe and needle were both sterilized by boiling in an instrument sterilizer before use. A 25-gauge needle was used for pigs weighing less than 300 gms., a 22-gauge needle for those between 300 and 500 gms., and a 20-gauge needle for animals weighing 500 gms. or more.

All samples of blood were allowed to stand at room temperature for one hour after drawing and then were placed in the refrigerator (7 - 9° C.) for twenty-four hours before the titer was determined. Each sample was centrifuged to obtain clear serum.

The hemolysin used was all from the same lot, obtained from Difco Laboratories and titrated before use. A unit was found to be 0.5 cc. of

a 1:4,000 dilution.

Corpuscles were obtained by bleeding a ewe from the jugular vein, using aseptic precautions. The blood was aspirated into a 30-cc. syringe and immediately expelled into a sterile flask containing glass beads. The flask was shaken for about ten minutes to defibrinate the blood, after which it was refrigerated. The defibrinated corpuscles were washed in saline the same day, three times or more, for ten minutes each time at 2200 r.p.m., packed by centrifuging for twenty minutes, made up to a 50 per cent suspension, and this suspension refrigerated and used within two days. The corpuscles were made up to a 2 per cent suspension in saline for use in the tests. The same three-year-old ewe was used throughout the investigation.

All dilutions were made with saline prepared by using 8.5 gms. of C.P. sodium chloride, making up to 1,000 cc. with cold tap water, and heating in flowing steam for thirty minutes. Only day-old saline was used throughout the work.

Complement Titration: The complement was titrated by determining the least amount necessary to bring about complete hemolysis of 0.5 cc. of a 2 per cent suspension of sheep cells in the presence of two units of hemolysin contained in 0.5 cc. The complement was diluted 1:10 and varying amounts were used, ranging from 0.06 to 0.26 cc. in 0.02-cc. intervals. The total volume in each dilution was made up to 3 cc. with saline. Saline, hemolysin, and complement controls were set up for each titration. Titers were recorded after a one-hour incubation in a water bath at 37° C. In Protocol 1 the arrangement used for each complement titration is shown.

PROTOCOL 1

Titration of Complement

| | Tube Number | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Complement 1:10 | cc. .06 | cc. .08 | cc. .10 | cc. .12 | cc. .14 | cc. .16 | cc. .18 | cc. .20 | cc. .22 | cc. .24 | cc. .26 | cc. .3 | cc. 0 | cc. 0 |
| Hemolysin 2 units | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | 0 | .5 | 0 |
| Sheep cells 2 per cent | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 | .5 |
| Saline | 1.94 | 1.92 | 1.9 | 1.88 | 1.86 | 1.84 | 1.82 | 1.8 | 1.78 | 1.76 | 1.74 | 2.2 | 2.0 | 2.5 |

Incubation in a water bath at 37° C. for 1 hour.

Animals: Animals were taken at random from a stock colony of guinea pigs of mixed weights and sexes. Those animals that gave evidence of pregnancy were avoided. The first twenty-two animals bled had feed up to the time of bleeding. Feed was given again after bleeding but was removed after several hours until blood was again obtained on the following day, a period of between eighteen and twenty-four hours intervening. Bleeding on two successive days was done to eliminate as far as possible any change in the titer of the complement due to factors other than feeding. With the last nine pigs the feed was removed prior to the first bleeding, but not before the second bleeding. A total of thirty-one complement samples was obtained from twenty-two males and nine females, with weights ranging from 350 to 1,000 gms. All animals were fed on a mixture of oats and wheat, various greens, and water. All of the animals tested were kept in the animal house.

RESULTS: An analysis of the results recorded in Table I will reveal that the average titer obtained when the animals' feed had been removed was 0.134 cc., whereas when the feed was not taken away, the average titer was 0.147 cc. This average difference of 0.013 cc. slightly favors

TABLE I

Complement Titers of a Group of Guinea Pigs With and Without Feed for Eighteen to Twenty-four Hours Before Bleeding

(Results expressed as cc. of 1:10 complement required to hemolyze 0.5 cc. of sheep cells with 2 units of hemolysin (0.5 cc. of 1:2,000 dilution) ).

| | | Titer | |
|---|---|---|---|
| Sex | Weight (grams) | With Feed | Without Feed |
| M. | 800* | .12 | .12 |
| M. | 1000 | .14 | .14 |
| M. | 1000 | .14 | .14 |
| M. | 900 | .14 | .16 |
| M. | 900 | .12 | .12 |
| M. | 650 | .12 | .08 |
| M. | 650 | .12 | .12 |
| M. | 650 | .14 | .16 |
| M. | 650 | .06 | .06 |
| M. | 650 | .10 | .12 |
| M. | 375 | .10 | .12 |
| M. | 375 | .12 | .10 |
| M. | 375 | .12 | .12 |
| F. | 375 | .20 | .20 |
| F. | 375 | .16 | .12 |
| F. | 375 | .20 | .20 |
| M. | 375 | .20 | .10 |
| F. | 375 | .12 | .12 |
| F. | 375 | .16 | .16 |
| M. | 700 | .12 | .10 |
| M. | 700 | .12 | .12 |
| M. | 700 | .14 | .16 |
| M. | 375 | .16 | .10 |
| F. | 375 | .18 | .18 |
| M. | 375 | .12 | .12 |
| F. | 375 | .22 | .16 |
| M. | 375 | .20 | .10 |
| M. | 375 | .20 | .16 |
| M. | 375 | .12 | .14 |
| F. | 375 | .16 | .14 |
| F. | 375 | .24 | .22 |
| Sum | | 4.56 | 4.16 |
| | Average difference = .013 ± .006 | | |

* Approximate weights

the removal of feed although the same titer occurred with fourteen animals, or 45.1 per cent. Eleven animals, or 35.5 per cent, gave a higher titer when without feed for eighteen to twenty-four hours before bleeding. However, in six animals, or 19.3 per cent, the complement was more hemolytic when the feed was not removed.

### 2. Complement Titers of Male and Non-Pregnant Female Guinea Pigs

Preparation of Materials: The blood samples for complement titration and the sheep cells, saline, and hemolysin necessary were prepared as described above (A. 1).

Complement Titration: The procedure was identical with that described above (A. 1).

Animals: All blood samples were obtained from animals from which feed had been removed for eighteen to twenty-four hours previously. Thirty-four males and twenty-eight females from the stock guinea pigs were bled. The weights varied from 250 to 1,300 gms. Females that were obviously pregnant were omitted from this determination. A grain mixture of oats and wheat, greens, and water constituted the ration fed. All of the animals were kept in the animal house. The data obtained are recorded in Table II.

RESULTS: The differences in titer observed were not significant, although the average difference of .012 cc. favored the male animals. By dividing the two sexes into weight groups, we find that fourteen males were in the 600 to 700 gms. group and fifteen in the 300 to 400 gms. group. This accounts for twenty-nine of thirty-four animals. The females were also divided, with seventeen in the 600 to 700 gms. group and ten in the 300 to 400 gms. group. The average titer for the 600 to 700 gms.

## TABLE II

Complement Titers of Male and Female Non-pregnant Guinea Pigs

(Results expressed as cc. of 1:10 complement required to hemolyze 0.5 cc. of sheep cells with 2 units of hemolysin (0.5 cc. of 1:2,000 dilution) ).

| Male | | Female | |
|---|---|---|---|
| Weight (grams) | Titer | Weight (grams) | Titer |
| 700* | .10 | 600* | .08 |
| 650 | .14 | 600 | .14 |
| 800 | .08 | 600 | .16 |
| 650 | .14 | 600 | .08 |
| 900 | .10 | 600 | .12 |
| 700 | .14 | 600 | .12 |
| 650 | .12 | 250 | .18 |
| 650 | .12 | 300 | .16 |
| 700 | .08 | 400 | .08 |
| 700 | .12 | 600 | .12 |
| 300 | .16 | 600 | .12 |
| 400 | .12 | 600 | .06 |
| 400 | .16 | 600 | .14 |
| 400 | .08 | 600 | .18 |
| 400 | .12 | 600 | .18 |
| 400 | .16 | 600 | .08 |
| 650 | .06 | 650 | .08 |
| 650 | .12 | 650 | .14 |
| 650 | .10 | 650 | .08 |
| 1000 | .14 | 650 | .08 |
| 675 | .10 | 375 | .20 |
| 675 | .08 | 375 | .16 |
| 900 | .12 | 375 | .20 |
| 675 | .12 | 375 | .12 |
| 1300 | .14 | 375 | .16 |
| 400 | .10 | 400 | .14 |
| 400 | .12 | 400 | .16 |
| 400 | .12 | 400 | .18 |
| 400 | .20 | | |
| 400 | .14 | | |
| 400 | .16 | | |
| 400 | .10 | | |
| 400 | .12 | | |
| 400 | .10 | | |
| Sum | 4.08 | | 3.70 |
| Mean | .12 | | .132 |

Average difference = .012 $\pm$ .0094

*Approximate weights

group was 0.110 cc. for males and 0.115 cc. for females. In the 300 to 400 gms. group, average titers of 0.130 and 0.156 cc. for males and females respectively were found. This may mean that the males, on an average, reach a higher titer sooner than the females. However, too few animals were used to make a definite statement. Had the number of animals in the lower weight group been equal in this experiment, the titers may have favored the males in a more significant manner. It should be mentioned, however, that the closeness of the complement titers in the 600 to 700 gm. group would indicate that had all animals for comparison been chosen of that weight, there would have been virtually no difference.

3. Comparison of Complement Titers of Sera from Guinea Pigs' Blood Pooled Before Clotting and Sera Pooled After Clotting

Preparation of Materials: The technique for bleeding the animals, dilution of hemolysin, preparation of sheep cells and saline was carried out as described above (A. 1).

Complement Titration: The complement titration was done as described above (A. 1).

Samples: Feed was removed for eighteen to twenty-four hours before bleeding. After 2 cc. of blood had been drawn from the guinea pig, 1 cc. was placed in an agglutination tube for mixing with the blood of two other animals, the pooling of these three bloods taking place before complete clotting. The pooled sample was allowed to remain at room temperature for one hour, and then placed in a refrigerator for twenty-four hours. The remaining 1 cc. of each sample was allowed to clot in a separate tube, remain for one hour at room temperature, and was then refrigerated for twenty-four hours. The sera from the same three animals pooled before bleeding were then pooled by taking 0.1 cc. of each serum and mixing thoroughly

before titration. Thirty-one samples of the above pooled sera were titrated.

Animals: The pooled blood comprised samples from males only, from females only, and mixtures of both as shown in Table III. Pregnant females were avoided. The guinea pigs were quartered in the animal house and fed greens, water, and a mixture of oats and wheat. Stock pigs were used that weighed from 350 to 1,000 gms.

RESULTS: It is the practice in some laboratories to bleed individual animals, allow the blood to clot, and pool the separate sera after twenty-four hours in the refrigerator followed by centrifuging. The results of this procedure compared with one in which the pooling was done at the time of bleeding are recorded in Table III.

Thirteen of the samples, representing 42 per cent, had equal titers; 45 per cent, or fourteen samples, had titers which favored pooling at the time of drawing the blood, and in only 13 per cent were the titers higher when the separate sera were pooled after twenty-four hours.

### 4. Effects of Physiological Salines on Hemolysin and Complement Titrations

Since the hemolytic activity of hemolysin and complement may vary at times because of the method of preparation or type of physiological saline used, several salines and methods of preparation were investigated.

#### a. Hemolysin Titration

Preparation of Materials: The hemolysin used in all of these experiments was of the same lot prepared by the Difco Laboratories and stated on the label to have a titer of 1:5,000 when used with 0.5 cc. of a 2 per cent suspension of corpuscles and 0.3 cc. of 1:30 complement.

TABLE III

A Comparison of Complement Titers of Sera From Guinea Pigs' Blood Pooled Before Clotting and Sera Pooled After Clotting

(Results expressed as cc. of 1:10 complement required to hemolyze 0.5 cc. of sheep cells with 2 units of hemolysin (0.5 cc. of 1:2,000 dilution) ).

| Sex | Weight (grams) | Titer | |
|---|---|---|---|
| | | Before | After |
| M.** | 1000* | .08 | .10 |
| M. | 1000 | .10 | .12 |
| M. | 350 | .14 | .16 |
| F. | 500 | .14 | .16 |
| F. | 500 | .14 | .14 |
| F. | 600 | .10 | .10 |
| F. | 525 | .14 | .16 |
| F. | 525 | .10 | .12 |
| F. | 600 | .16 | .18 |
| F. | 500 | .14 | .14 |
| M.M.F. | 350 | .12 | .14 |
| F.F.M. | 350 | .12 | .14 |
| M.M.F. | 700 | .14 | .14 |
| M.M.F. | 700 | .16 | .16 |
| F.F.M. | 700 | .10 | .12 |
| F. | 650 | .16 | .16 |
| M. | 650 | .16 | .16 |
| M. | 650 | .16 | .16 |
| M. | 600 | .14 | .14 |
| M. | 350 | .10 | .16 |
| F. | 350 | .12 | .16 |
| M. | 350 | .12 | .14 |
| F. | 600 | .16 | .14 |
| F. | 600 | .12 | .14 |
| F. | 750 | .10 | .10 |
| M. | 400 | .16 | .14 |
| F. | 400 | .16 | .16 |
| F.F.M. | 400 | .14 | .14 |
| M.M.F. | 400 | .16 | .12 |
| M.M.F. | 400 | .18 | .16 |
| F.F.M. | 400 | .18 | .18 |
| Sum | | 4.20 | 4.44 |

Average difference = .0077 $\pm$ .00343

*Approximate weights

** Three guinea pigs per sample

Complement and corpuscles were secured and prepared as described under "Preparation of Materials" A. 1. Complement was pooled before complete clotting and was obtained from healthy guinea pigs whose titers were already known to be satisfactory. The feed was removed for eighteen to twenty-four hours before bleeding. Complement was used the same day (i.e. after refrigeration for twenty-four hours) except in the last complement experiment when it was used the second day.

Saline solutions were prepared with 0.85 per cent C.P. sodium chloride made up in various waters, and of these some were used unheated, some were placed in flowing steam for thirty minutes, and others autoclaved for thirty minutes. Determinations were made of the pH of these solutions just after use, employing a quinhydrone electrode at first, and later a colorimetric method with standards and indicator solutions that had recently been checked.

A modified Ringer's solution consisting of 8.0 gms. of sodium chloride, 0.14 gm. of potassium chloride, 0.12 gm. of calcium chloride, and 0.20 gm. of sodium bicarbonate per liter of distilled water was used in the first two series of hemolysin titrations. The various salines prepared were used in these series for dilution of the 1:100 hemolysin and for equalizing the volumes. A tap water saline which had been steamed was used for making the first 1:100 dilution of hemolysin and also for diluting the complement and corpuscles.

In the second two series, a phosphate-buffered saline like Mason and Sanford's (34), except that it was made with single distilled water, was used. Another tap water was secured from Washington, D. C., since that tap water is of relatively high chemical purity, as shown by the fact

that it is used in batteries, and was of somewhat different character than other tap water available. The various salines prepared were used in these series for the entire dilution of glycerinated hemolysin and for equalizing the volumes. An autoclaved tap water was used in these last two series for dilution of the complement and corpuscles. In Series IV the saline solutions used were those used the preceding day in Series III.

Titration of Hemolysin: In Protocol 2 the arrangement used for each titration of hemolysin with each saline is shown (except additional intermediate dilutions of 1,500 - 5,500 which were made in the last experiment).

PROTOCOL 2

Titration of Hemolysin

| | Tube Number | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| | cc. | cc. | cc. | cc. | cc. | cc. | cc. |
| Hemolysin (diluted) | .5 | .5 | .5 | .5 | .5 | .5 | .5 |
| Complement 1:10 | .1 | .1 | .1 | .1 | .1 | .1 | .1 |
| Corpuscles 2 per cent | .5 | .5 | .5 | .5 | .5 | .5 | .5 |
| Saline | 1.9 | 1.9 | 1.9 | 1.9 | 1.9 | 1.9 | 1.9 |
| Incubation in a water-bath at 37° C. for 1 hour | | | | | | | |
| Results | | | | | | | |
| Hemolysin dilution | 1:1,000 | 2,000 | 3,000 | 4,000 | 5,000 | 6,000 | 7,000 |

RESULTS: The results of the first series in hemolysin titration (tabulated in Table IV) showed a decidedly greater titer when using saline prepared from tap water (1:3,000 or 4,000) compared with the use of saline prepared with distilled water (1:1,000). A higher titer was also indicated in those salines that had been placed in flowing steam

TABLE IV

Effect on Hemolysin Titration of Salines Prepared from Distilled, Tap, or Buffered Waters - Unheated, Steamed or Autoclaved

| Saline* | | Series I Titer | I pH | Series II Titer | II pH | Series III Titer | III pH** | Series IV Titer | IV pH** |
|---|---|---|---|---|---|---|---|---|---|
| Distilled | | | | | | | | | |
| | DU | 1000 | 5.6 | 1000- | 5.6 | 2000 | 6.2 | 1500 | 6.4 |
| | DS | 1000+ | 6.1 | 1000+ | 6.0 | 2000 | 6.3 | 2000 | 6.4 |
| | DA | | | | | 2000 | 6.3 | 2000 | 6.4 |
| Tap | | | | | | | | | |
| | TU | 3000 | 7.1 | 2000- | 7.0 | 3000 | 7.4 | 2500 | 7.4 |
| | TS | 4000+ | 7.3 | 2000+ | 7.0 | 3000 | 7.4 | 3000 | 7.4 |
| | TA | | | | | 4000+ | 7.6 | 4000 | 7.4 |
| Tap-Ringer's | | | | | | | | | |
| | TRU | 1000 | 7.9 | 1000- | 7.9 | | | | |
| | TRS | 4000 | 7.8 | 4000 | 7.8 | | | | |
| Distilled-phosphates | DPU | | | | | 3000 | 7.1 | 3000 | 7.1 |
| | DPS | | | | | 3000 | 7.3 | 3000 | 7.3 |
| | DPA | | | | | 3000 | 7.3 | 3500 | 7.3 |
| Tap (Washington) | | | | | | | | | |
| | TWU | | | | | 4000+ | 7.8 | 4500 | 8.0 |
| | TWS | | | | | 4000+ | 8.0 | 5000 | 8.0 |
| | TWA | | | | | 4000+ | 8.2 | 5000 | 8.2 |

*Complement and corpuscles were diluted with tap water saline, steamed in I, II and autoclaved in III, IV.

**Colorimetric pH

A, autoclaved
D, distilled
P, phosphates
R, Ringer's
S, steam (flowing)
T, tap
U, unheated
W, Washington
- not complete
+ nearly complete in next higher dilution

for thirty minutes compared with those unheated. The pH of the solutions also varied with the titer with the unbuffered salines, whereas, with a modified Ringer's solution, there was no correlation of pH and hemolysin titer. Ringer's solution steamed had a titer equal to that of the solution made with steamed tap water, but unheated Ringer's solution gave a very low titer corresponding to that of a solution made with distilled water.

Repetition of this experiment after two days with freshly prepared salines gave comparable results as shown by Series II. In this case the titer with tap water (both steamed and unheated) was not so high as with Ringer's solution (steamed), and likewise the pH of the tap water was not so high as in the preceding series.

Other experiments were then conducted on complement titrations but after a month, Series III and IV were performed, discontinuing Ringer's solution, adding a phosphate-buffered solution (Mason and Sanford's) and another tap water, and including an autoclaved saline in each series. Series IV was performed with the same reagents used the preceding day in Series III but with a series of intermediate dilutions to allow a more accurate record of titer. In both series distilled water saline was again lowest in titer; distilled water buffered with phosphates was considerably higher in titer than when unbuffered, and but little below the tap water, and the other tap water used now for the first time was highest of all. The autoclaved salines usually gave higher titers than the unheated salines, the salines heated in flowing steam sometimes being of the same titer as autoclaved salines and sometimes lower, even as low as the unheated salines. In all cases the pH values of the same salines, heated or unheated, were

nearly the same and there was again a correlation of pH with the titers obtained.

b. Complement Titration

Preparation of Materials: The reagents used were prepared as indicated under hemolysin titration. A solution similar to Brooks' (7) Ringer-Tyrode's solution was also prepared. This formed considerable precipitate with tap or distilled water so that the solution was unfit for use because the precipitate obscured the results.

Titration of Complement: The arrangement used for each titration of complement for each saline was done as shown in Protocol 1 except that amounts of 0.28 and 0.30 cc. of complement were added. In the experiments themselves the various salines were used for only one reagent in each test, the other reagents in each case being prepared with tap water saline heated in flowing steam for thirty minutes, which was also used for preparing the 1:100 dilution of hemolysin. The results obtained are tabulated in Table V.

A further series of experiments were conducted in which the varying salines were used for each reagent in the test, no other saline being used in any place with the exception* of the preliminary washing of the blood corpuscles and their dilution to a 50 per cent suspension. In addition, the effect of the addition of magnesium chloride was tested with one distilled and one tap water. These results are presented in Table VI.

RESULTS: In the three trials of complement titration in which the volume alone was made up with varying salines (Table V)(the amount varying from 1.7 to 1.9 cc.), distilled water was poorer than tap in one, about

*Tap water saline steamed for thirty minutes.

TABLE V

Effect on Complement Titration of Salines Prepared from Distilled, Tap, or Buffered Distilled Water - Unheated, Steamed or Autoclaved

(Salines used for only one reagent. Results expressed as cc. of 1:10 complement required)

| Series | Saline* | | Volume (1.7 - 1.9 cc.) | Corpuscles (0.5 cc.) | Hemolysin (0.5 cc.) | Complement (0.09-0.18 cc.) | pH |
|---|---|---|---|---|---|---|---|
| I | Distilled | DU | 0.18 | 0.16 | 0.12 | 0.14 | 5.5 |
| | | DS | 0.18 | 0.14 | 0.14 | 0.14 | 5.8 |
| | | DA | 0.18 | 0.10 | 0.14 | 0.14 | 5.8 |
| | Tap | TU | 0.14 | 0.18 | 0.18 | 0.14 | 6.7 |
| | | TS | 0.12 | 0.14 | 0.18 | 0.14 | 6.9 |
| | | TA | 0.10 | 0.12 | 0.18 | 0.14 | 7.1 |
| II | Distilled | DU | 0.10 | 0.18 | 0.12 | 0.08 | 5.5 |
| | | DS | 0.10 | 0.14 | 0.14 | 0.08 | 5.6 |
| | | DA | 0.10 | 0.12 | 0.14 | 0.08 | 5.9 |
| | Tap | TU | 0.14 | 0.16 | 0.14 | 0.08 | 6.4 |
| | | TS | 0.12 | 0.14 | 0.14 | 0.08 | 6.6 |
| | | TA | 0.12 | 0.12 | 0.14 | 0.08 | 6.8 |
| | Distilled-phosphates | DPU | 0.10 | 0.12 | 0.10 | 0.10 | 6.6 |
| | | DPS | 0.08 | 0.10 | 0.12 | 0.10 | 6.9 |
| | | DPA | 0.08 | 0.10 | 0.10 | 0.10 | 6.9 |
| III | Distilled | DU | 0.16 | 0.22 | 0.18 | 0.18 | 6.0 |
| | | DS | 0.16 | 0.20 | 0.18 | 0.18 | 6.0 |
| | | DA | 0.16 | 0.18 | 0.18 | 0.18 | 6.3 |
| | Tap | TU | 0.18 | 0.20 | 0.18 | 0.18 | 6.8 |
| | | TS | 0.18 | 0.18 | 0.18 | 0.18 | 7.1 |
| | | TA | 0.16 | 0.16 | 0.18 | 0.16 | 7.1 |
| | Distilled-phosphates | DPU | 0.14 | 0.22 | 0.18 | 0.20 | 6.7 |
| | | DPS | 0.14 | 0.22 | 0.18 | 0.18 | 6.9 |
| | | DPA | 0.16 | 0.18 | 0.20 | 0.18 | 6.9 |

* A tap water saline (steamed) was used in preparing all other reagents except the one being tested (including the 1:100 hemolysin).

A, autoclaved
D, distilled
P, phosphates
S, steam (flowing)
T, tap
U, unheated

## TABLE VI

**Effect on Complement Titration of Salines Prepared from Distilled, Tap or Treated Waters - Unheated, Steamed or Autoclaved**

(Salines used for each reagent. Results expressed as cc. of 1:10 complement required)

| Saline* | | Complement | pH** |
|---|---|---|---|
| | | cc. | |
| Distilled | | | |
| | DU | 0.16 | 6.6 |
| | DS | 0.20 | 6.4 |
| | DA | 0.18 | 6.6 |
| Tap | | | |
| | TU | 0.14 | 7.6 |
| | TS | 0.14 | 7.6 |
| | TA | 0.16 | 7.6 |
| Distilled-phosphates | | | |
| | DPU | 0.08 | 7.3 |
| | DPS | 0.10 | 7.3 |
| | DPA | 0.10 | 7.3 |
| Distilled-magnesium | | | |
| | DMU | 0.08 | 6.6 |
| | DMS | 0.08 | 6.5 |
| | DMA | 0.08 | 6.7 |
| Tap-magnesium | | | |
| | TMU | 0.10 | 7.5 |
| | TMS | 0.12 | 7.5 |
| | TMA | 0.10 | 7.8 |

*Diluent same for each reagent and for equalizing volume
**Colorimetric pH

A, autoclaved
D, distilled
M, magnesium chloride
P, phosphates
S, steam (flowing)
T, tap
U, unheated

the same in one, and better in one trial. The addition of phosphates to distilled water improved the titer slightly under these conditions. The improvement in titer due to heating was also less striking in these experiments, compared to titration of hemolysin, the heated salines giving higher titers in four instances, the same as unheated in three and in one case the autoclaved saline was slightly poorer than the unheated.

When the corpuscles alone were diluted with the various salines (0.5 cc. being used in each tube), the distilled water gave about the same titer as tap water, and the addition of phosphates improved the titer in one series and did not in the other series used. However, there was a noticeable relatively uniform increase in titer of steamed over unheated salines and of autoclaved over steamed salines. It would seem that the increased evaporation of water and accompanying concentration of salts due to heating the saline may affect the red cells to such an extent that the complement titer was correspondingly improved.

When hemolysin alone was diluted further than 1:100 with various salines (0.5 cc. being added) no influence was evident. There was little difference between distilled water and tap water and (except in one case) phosphate-buffered salines. In the first series the difference between the distilled water and tap water salines might be significant. Likewise, there was no apparent effect of heated compared with unheated waters.

The effect of varying the saline in the dilution of the complement alone was striking in the almost completely identical titers regardless of the salines or of their exposure to heat. Only 0.09 to 0.18 cc. of saline would have been used in any test, and in a volume of 3 cc. containing

a suitable saline such as heated tap water (used in these experiments), the influence of the one variable added in such a small amount was negligible. This may account for the similar results obtained with hemolysin, for although 0.5 cc. was used, the first 1:100 dilution was made with a steamed tap water saline.

The actual titers obtained in the three series vary, of course, due to different complement being used. There was apparently no correlation of complement titer and pH, unless it was in the cases where there was an effect due to heating, as here the pH also was slightly higher in the heated salines.

When the same saline was used as the only diluent for a complement titration (Table VI), saline made with our distilled water required a larger amount of complement than did that made with our tap water. The addition of phosphates to the distilled water reduced by about half the amount of complement needed in this experiment. The addition of magnesium chloride to our distilled water likewise reduced the amount of complement needed to less than half; and even when added to our tap water the amount of complement needed was considerably reduced.

The difference between heated and unheated distilled and tap water was not so great in the last experiments on complement titration as with hemolysin titration. It should be noted, however, the pH of the two types of saline was near neutrality and there was not the increase in pH with the increase in heating usually found in preceding experiments. The pH of unheated solutions was as high or in some cases higher than the heated, which may account for the small discrepancies in titer between the unheated and the heated salines. Buffered solutions showed no correlation of titers obtained between the heated or unheated salines.

## 5. Comparison of Surface Tension Measurement and Hemolytic Activity of Guinea Pig Complement

Heat applied to sera causes an alteration of colloidal state which is measurable by a lowering of surface tension. Exposure of sera to ultra-violet light will also cause a drop in surface tension. The injection of foreign proteins, bacteria, and gum shellac is reported to bring about an increased time drop over that noted in normal animals, probably through some change in physicochemical equilibrium within the serum, and probably not due to the presence of antibodies. Therefore, when guinea pigs show a variation in complement potency, this might be correlated with a change in surface tension, and a physical measurement such as surface tension might be employed to indicate the hemolytic strength of guinea pig complement.

Preparation of Materials: Samples of complement and corpuscles were obtained and prepared as described under "Preparation of Materials" A. 1. The hemolysin, which had a titer of 1:6,000, was obtained from the Difco Laboratories.

Complement Titration: The complement titration was done as indicated in Protocol 1 except that amounts of 0.28 and 0.30 cc. of complement were added. The hemolytic titer is recorded in the results as a dilution of 1:10. The saline for these titrations was prepared by using 8.5 gms. of C.P. sodium chloride and 0.1 gm. of magnesium chloride, making up to a liter with cold tap water, and autoclaving for thirty minutes at fifteen pounds' pressure.

The sera were diluted for surface tension measurements with a 0.9 per cent salt solution, made with C.P. sodium chloride in distilled water.

All glassware used in measuring surface tension was soaked for several days in cleaning solution, thoroughly rinsed in distilled water, and dried before use.

Surface Tension Determination: The du Noüy tensiometer (38) was used for measuring surface tension. This instrument measures the force necessary to remove a platinum-iridium ring from the surface of a liquid. The stress necessary to tear off the film formed around the ring is measured by the torsion of a steel wire. The degree of torsion is read from a dial connected to the wire and this is converted to a surface tension reading in dynes by first obtaining a reading on distilled water and using a simple calculation.

Surface tension determinations were made by placing 1.5 cc. of the diluted serum on a watch glass which had a diameter of 7.5 cm. All measurements were made in about twenty seconds. After the initial reading, the watch glasses were set aside for two hours before the second or static reading was made. Subtracting the first reading from the second gave the time drop. The determinations were made in a small room free from such disturbing elements as air currents and jarring of samples. The samples of sera were titrated and the surface tension determined after an interval of not more than six hours.

Preliminary surface tension measurements using undiluted serum and dilutions of 1:10, 1:100, 1:1,000, 1:5,000, 1:10,000, and 1:100,000 showed the greatest time drop was at a dilution of 1:10,000. Therefore, all time drops are reported on dilutions of 1:10,000 at the end of a two-hour period. This is also in agreement with du Noüy's findings on normal and immune sera. When serum is diluted with physiological salt solution the decrease in surface energy is less rapid than in pure serum. Equilibrium is reached in approximately two hours. All measurements were made at room temperature which varied between 21° and 23° C. Between ten and fifteen samples were run at one time.

Samples: Samples of sera were obtained from pregnant females, non-pregnant females, and from males. The weights of the animals varied between 250 and 900 gms. Only normal, unused guinea pigs were bled. du Noüy (40, 41) showed that the formation of antibodies was attended by a larger time drop than was found in normal animals. With rabbits assumed to be normal, time drops varying between 6 and 18 dynes were noted, but all showing greater drops than 10 dynes were eliminated from further tests. The elimination of such animals was not undertaken in this experiment. Feed was removed for eighteen hours before bleeding to avoid chylous sera and only clear sera were used. Samples 1 to 27 were sera of males, the next 18 were taken from females that were not obviously pregnant, and the last 7 samples of sera were from pregnant animals.

RESULTS: The surface tension measurements and hemolytic titers of 52 guinea pig sera are presented in Table VII. In Table VIII, the males, females, and pregnant animals are again separated but within these, the sera are grouped according to titer. Only the time drops and the titers are tabulated.

Eleven of the 27 males showed a titer of 0.10 cc. and the time drop varied from 6.3 dynes to 14.3. The six sera which had a titer of 0.08 cc. showed time drops varying from the highest which was 11.2 to the lowest of 7.4 dynes. Four samples of sera with a titer of 0.12 cc. had time drops varying between 5.3 and 9.0 dynes. Two sera had time drops between 12 and 13 dynes, with titers of 0.06 cc. Two others titered 0.14 cc. and the time drops were 11.4 and 8.8 dynes. One serum sample titered 0.16 cc. and another 0.18 cc. with time drops of 11.1 and 8.1 dynes respectively.

TABLE VII

Comparison of Time Drop and Titer of Guinea Pig Sera

| Serum | Initial Surface Tension | Static Surface Tension | Time Drop | Titer |
|---|---|---|---|---|
| | dynes | dynes | dynes | |
| 1 | 69.3* | 59.6 | 9.7 | 0.08* |
| 2 | 72.0 | 64.9 | 7.1 | 0.10 |
| 3 | 68.9 | 60.5 | 8.4 | 0.12 |
| 4 | 68.3 | 59.3 | 9.0 | 0.12 |
| 5 | 70.2 | 62.0 | 8.2 | 0.12 |
| 6 | 68.0 | 59.2 | 8.8 | 0.14 |
| 7 | 69.3 | 61.3 | 8.0 | 0.08 |
| 8 | 70.3 | 64.0 | 6.3 | 0.10 |
| 9 | 66.6 | 61.3 | 5.3 | 0.12 |
| 10 | 64.1 | 54.8 | 9.3 | 0.10 |
| 11 | 69.0 | 62.0 | 7.0 | 0.10 |
| 12 | 70.0 | 58.9 | 11.1 | 0.16 |
| 13 | 68.0 | 59.9 | 8.1 | 0.18 |
| 14 | 70.0 | 58.6 | 11.4 | 0.14 |
| 15 | 72.3 | 63.9 | 8.4 | 0.10 |
| 16 | 71.0 | 58.6 | 12.4 | 0.06 |
| 17 | 70.8 | 57.6 | 13.2 | 0.10 |
| 18 | 65.1 | 56.6 | 8.5 | 0.10 |
| 19 | 69.4 | 58.6 | 10.8 | 0.08 |
| 20 | 66.7 | 59.3 | 7.4 | 0.08 |
| 21 | 68.1 | 58.6 | 9.5 | 0.10 |
| 22 | 68.9 | 54.9 | 14.0 | 0.10 |
| 23 | 69.2 | 56.3 | 12.9 | 0.06 |
| 24 | 68.1 | 56.9 | 11.2 | 0.08 |
| 25 | 66.7 | 54.6 | 12.1 | 0.10 |
| 26 | 66.2 | 55.2 | 11.0 | 0.08 |
| 27 | 69.8 | 55.5 | 14.3 | 0.10 |
| 28 | 69.0 | 56.7 | 12.3 | 0.14 |
| 29 | 69.4 | 56.7 | 12.7 | 0.16 |
| 30 | 69.5 | 54.4 | 15.1 | 0.20 |
| 31 | 68.0 | 58.0 | 10.0 | 0.12 |
| 32 | 66.6 | 54.1 | 12.5 | 0.18 |
| 33 | 72.4 | 64.6 | 7.8 | 0.20 |
| 34 | 72.3 | 62.0 | 10.0 | 0.26 |
| 35 | 69.0 | 59.0 | 9.6 | 0.20 |
| 36 | 69.0 | 55.6 | 13.4 | 0.10 |
| 37 | 67.9 | 59.9 | 8.0 | 0.10 |
| 38 | 70.0 | 59.6 | 10.4 | 0.18 |
| 39 | 67.6 | 59.6 | 7.7 | 0.10 |
| 40 | 68.0 | 55.9 | 12.1 | 0.24 |
| 41 | 66.4 | 53.9 | 12.5 | 0.24 |
| 42 | 71.2 | 55.3 | 15.9 | 0.22 |
| 43 | 72.9 | 54.3 | 18.6 | 0.22 |
| 44 | 69.4 | 52.6 | 16.8 | 0.14 |
| 45 | 65.9 | 57.3 | 8.6 | 0.20 |

TABLE VII
(continued)

| Serum | Initial Surface Tension | Static Surface Tension | Time Drop | Titer |
|---|---|---|---|---|
| | dynes | dynes | dynes | |
| 46 | 71.9 | 54.1 | 17.0 | 0.30+ |
| 47 | 66.6 | 57.6 | 9.0 | 0.30 |
| 48 | 70.0 | 57.4 | 12.6 | 0.26 |
| 49 | 70.0 | 60.4 | 9.6 | 0.22 |
| 50 | 67.4 | 54.8 | 12.6 | 0.20 |
| 51 | 70.4 | 58.4 | 12.0 | 0.22 |
| 52 | 68.2 | 53.5 | 14.7 | 0.18 |

Nos. 1 - 27 all males; 28 - 45 females; 46 - 52 pregnant females

*Surface tension measurements were made with sera dilutions 1:10,000; titrations with 1:10 dilutions.

TABLE VIII

Comparison of Time Drop and Titer of Guinea Pig Sera
Arranged According to Titer

| Males | | | Females (non-pregnant) | | | Females (pregnant) | | |
|---|---|---|---|---|---|---|---|---|
| Serum | Time Drop dynes | Titer | Serum | Time Drop dynes | Titer | Serum | Time Drop dynes | Titer |
| 16 | 12.4* | 0.06* | 39 | 7.7* | 0.10* | 52 | 14.7* | 0.18* |
| 23 | 12.9 | 0.06 | 37 | 8.0 | 0.10 | | | |
| | | | 36 | 13.4 | 0.10 | 50 | 12.6 | 0.20 |
| 20 | 7.4 | 0.08 | | | | | | |
| 7 | 8.0 | 0.08 | 31 | 10.0 | 0.12 | 49 | 9.6 | 0.22 |
| 1 | 9.7 | 0.08 | | | | 51 | 12.0 | 0.22 |
| 19 | 10.8 | 0.08 | 28 | 12.3 | 0.14 | | | |
| 26 | 11.0 | 0.08 | 44 | 16.8 | 0.14 | 48 | 12.6 | 0.26 |
| 24 | 11.2 | 0.08 | | | | | | |
| | | | 29 | 12.7 | 0.16 | 47 | 9.0 | 0.30 |
| 8 | 6.3 | 0.10 | | | | 46 | 17.0 | 0.30* |
| 11 | 7.0 | 0.10 | 38 | 10.4 | 0.18 | | | |
| 2 | 7.1 | 0.10 | 32 | 12.5 | 0.18 | | | |
| 15 | 8.4 | 0.10 | | | | | | |
| 18 | 8.5 | 0.10 | 33 | 7.8 | 0.20 | | | |
| 10 | 9.3 | 0.10 | 45 | 8.6 | 0.20 | | | |
| 21 | 9.5 | 0.10 | 35 | 9.6 | 0.20 | | | |
| 25 | 12.1 | 0.10 | 30 | 15.1 | 0.20 | | | |
| 17 | 13.2 | 0.10 | | | | | | |
| 22 | 14.0 | 0.10 | 42 | 15.9 | 0.22 | | | |
| 27 | 14.3 | 0.10 | 43 | 18.6 | 0.22 | | | |
| | | | | | | | | |
| 9 | 5.3 | 0.12 | 40 | 12.1 | 0.24 | | | |
| 5 | 8.2 | 0.12 | 41 | 12.5 | 0.24 | | | |
| 3 | 8.4 | 0.12 | | | | | | |
| 4 | 9.0 | 0.12 | 34 | 10.0 | 0.26 | | | |
| | | | | | | | | |
| 6 | 8.8 | 0.14 | | | | | | |
| 14 | 11.4 | 0.14 | | | | | | |
| | | | | | | | | |
| 12 | 11.1 | 0.16 | | | | | | |
| | | | | | | | | |
| 13 | 8.1 | 0.18 | | | | | | |

*Surface tension measurements were made with sera dilutions of 1:10,000; titrations with 1:10 dilutions.

As a whole, the titers of the non-pregnant females were lower than those of the males (Table VIII). This may be because the females were selected from smaller animals. The highest titer obtained from females was 0.10 cc. and the lowest 0.26 cc. The eighteen samples were so distributed among these titers that no one group predominated. The largest group had a titer of 0.20 cc. and contained four samples. The time drop varied between 7.8 and 15.1 dynes. Drops varying from 7.7 to 13.4 were obtained on three samples of complement with a titer of 0.10 cc. The following groups had two samples each: 0.14 cc., 0.18 cc., 0.22 cc., and 0.24 cc., with differences in time drop between the two samples of each group amounting to 4.5, 2.1, 2.7, and 0.4 dynes respectively.

While pregnancy definitely influenced the titer of guinea pig complement the time drops were no greater in some instances than those recorded for non-pregnant females or for males. However, a large percentage of the sera from females had higher time drops than those from males.

Table VII reveals one significant fact; a larger percentage of males showed time drops of less than 10 dynes than was recorded for either pregnant or non-pregnant females. Sixty per cent of the males showed a drop of less than 10 dynes while only approximately 27 and 30 per cent of non-pregnant and pregnant females respectively gave the same results as the males.

## B. Factors Influencing the Production of Guinea Pig Complement of Satisfactory Titer

Published reports show a rather wide variation in their requirements regarding the care and management of guinea pigs necessary to yield suitable complement. Accordingly, a number of factors were investigated in order to determine their possible effect upon the production of complement of high titer. These include feed, sex, age, weight, difference in litter mates, and the influence of temperature, and of sunlight.

Preparation of Materials: The samples of sera and sheep cells were obtained and prepared, the hemolysin diluted, and saline made as described under "Preparation of Materials" A. 1. The animals were bled and sera titrated monthly except in the first month when it was done twice. Feed was removed for eighteen to twenty-four hours before bleeding.

Complement Titration: Each sample of complement was titrated according to the arrangement in Protocol 1. All mention made hereafter of complement titers refers to dilutions of 1:10.

Animals: Nine different litters of guinea pigs were received on June 4, 1935 to which were later added three more litters to replace animals that had died. From the variation in weights at the time of receipt of the animals listed in Table IX, it is clear that they vary somewhat in age.

The weights of only litters 5 and 9 would indicate them to be over three weeks old since pigs at birth will vary from approximately 40 gms. to 90 gms., depending upon the number per litter and the age of the mothers. This experiment extended over one year, the first titration being made on June 14, 1935, and the last on June 6, 1936.

## TABLE IX

### A Comparison of Complement Titers, Age, and Weight of Guinea Pigs Housed Outdoors and Indoors on Different Rations

(Results of titration expressed as cc. of 1:10 complement required to hemolyze 0.5 cc. of sheep cells with 2 units of hemolysin (0.5 cc. of 1:2,000 dilution))

| Cage | | Guinea Pig | | | | Date of Determining Titer and Weight | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 1935 | | | | | | | | 1936 | | | | |
| Number and Location | Feed | Number | Litter Number | Sex | Weight at Start | 6/14 | 6/29 | 7/30 | 8/31 | 10/2 | 11/1 | 12/3 | 12/31 | 1/30 | 2/29 | 3/28 | 5/2 | 6/6 |
| 1 Outside | Wheat Oats Greens | 409 | 1 | M | | .18 | .24 | .22 | .18 | .22 | .18 | .10 | .10 | .12 | .10 | .08 | .12 | .14 |
| | | | | | 90 | 95 | 120 | 231 | 356 | 459 | 532 | 545 | 535 | 576 | 622 | 660 | 653 | 641** |
| | | 198 | 9 | M | | .18 | .18 | .22 | .24 | .20 | .16 | .10 | .10 | .10 | .08 | .08 | .10 | .10 |
| | | | | | 274 | 272 | 302 | 428 | 554 | 673 | 762 | 772 | 775 | 862 | 947 | 930 | 1002 | 1013 |
| | | 408 | 10 | M | | | | | .26+ | .26+ | .20 | .18 | .10 | .12 | .10 | .12 | .10 | .10 |
| | | | | | 90 | | | | 124 | 218 | 312 | 382 | 445 | 510 | 559 | 628 | 636 | 615 |
| | | 410 | 3 | F | | .26+ | - | .22 | .16 | .16 | .20 | .14 | .16 | .24 | .18 | .26 | .26+ | .26 |
| | | | | | 104 | 105 | 122 | 232 | 376 | 502 | 637 | 611 | 654 | 684 | 704 | 763 | 1004 | 710 |
| | | 187 | 4 | F | | .16 | .16 | .20 | .20 | .22 | .18 | .22 | .12 | .22 | .12 | .26 | .18 | .24 |
| | | | | | 142 | 144 | 164 | 259 | 355 | 428 | 506 | 628 | 494 | 533 | 633 | 706 | 598 | 646 |
| 1 Inside | Wheat Oats Greens | 177 | 1 | M | | .22 | .24 | .22 | .20 | .16* | | | | | | | | |
| | | | | | 92 | 104 | 120 | 244 | 370 | 457 | | | | | | | | |
| | | 194 | 7 | M | | .22 | .20 | .22 | .22 | .24 | .16 | .16* | | | | | | |
| | | | | | 136 | 160 | 198 | 336 | 448 | 526 | 600 | 626 | | | | | | |
| | | 301 | 12 | M | | | | | .26+ | .26+ | .18 | .10 | .08* | | | | | |
| | | | | | 81 | | | | 120 | 200 | 289 | 376 | 463 | | | | | |
| | | 406 | 4 | F | | .16 | .18 | .16 | .18 | .26+ | .20 | .20 | .26 | .20 | .14 | .12 | .10 | .20 |
| | | | | | 190 | 203 | 236 | 368 | 533 | 637 | 558 | 595 | 718 | 846 | 937 | 1024 | 1062 | 1101 |
| | | 191 | 6 | F | | .24 | .24 | .22 | .20 | .22 | .14 | .12 | .16 | .26+ | .12 | .12 | .18 | .14 |
| | | | | | 86 | 98 | 119 | 260 | 418 | 553 | 468 | 535 | 726 | 639 | 636 | 710 | 764 | 825 |

**TABLE IX**
**(continued)**

| Cage | | Guinea Pig | | | | Date of Determining Titer and Weight | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 1935 | | | | | | | | 1936 | | | |
| Number and Location | Feed | Number | Litter Number | Sex | Weight at Start | 6/14 | 6/29 | 7/30 | 8/31 | 10/2 | 11/1 | 12/3 | 12/31 | 1/30 | 2/29 | 3/28 | 5/2 | 6/6 |
| 2 Outside | Pellets Oats Cabbage | 418 | 2 | M | | .20 | .20 | .18 | .20 | .20 | .18 | .10 | .10 | .08 | .10 | .10 | .12 | .14 |
| | | | | | 95 | 123 | 184 | 327 | 470 | 556 | 608 | 638 | 624 | 612 | 658 | 713 | 715 | 668 |
| | | 413 | 8 | M | | .22 | .22 | .20 | .20 | .12 | .16 | .14 | .10 | .14 | .12 | .12 | .12 | .10 |
| | | | | | 107 | 140 | 186 | 321 | 468 | 541 | 680 | 642 | 597 | 623 | 742 | 777 | 812 | 859 |
| | | 178 | 1 | F | | .26+ | .26+ | .18 | .24 | .12 | .16 | .16 | .20 | .12 | .26 | .24 | .20 | .26+ |
| | | | | | 78 | 109 | 156 | 282 | 439 | 513 | 634 | 685 | 808 | 580 | 860 | 632 | 717 | 866 |
| | | 403 | 5 | F | | .20 | .16 | .20 | .20 | .20 | .12 | .12 | .22 | .10 | .20 | .22 | .24 | .26 |
| | | | | | 206 | 254 | 300 | 442 | 572 | 644 | 745 | 828 | 1064 | 761 | 960 | 802 | 927 | 1200 |
| 2 Inside | Pellets Oats Cabbage | 186 | 4 | M | | .20* | | | | | | | | | | | | |
| | | | | | 135 | 167 | | | | | | | | | | | | |
| | | 189 | 5 | M | | .24 | .20 | .18 | .18 | .22 | .16 | .16 | .22 | .10 | .10 | .08 | .08 | .12 |
| | | | | | 180 | 222 | 302 | 492 | 622 | 733 | 818 | 813 | 824 | 854 | 910 | 958 | 961 | 1017 |
| | | 195 | 7 | M | | .20 | .24 | .20 | .24 | .24 | .16 | .16 | .14 | .10 | .10 | .10 | .14 | .16 |
| | | | | | 126 | 167 | 226 | 402 | 555 | 675 | 774 | 744 | 758 | 752 | 838 | 891 | 887 | 943 |
| | | 420 | 12 | M | | | | | .24 | .20 | .16 | .14 | .12 | .12 | .12 | .10 | .12 | .10 |
| | | | | | 120 | | | | 166 | 372 | 502 | 528 | 564 | 639 | 701 | 732 | 740 | 825 |
| | | 180 | 2 | F | | .22 | .20 | .12 | .14 | .14 | .24 | .12* | | | | | | |
| | | | | | 85 | 126 | 198 | 344 | 512 | 677 | 922 | 668 | | | | | | |

TABLE IX
(Continued)

| Cage | | Guinea Pig | | | | Date of Determining Titer and Weight | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 1935 | | | | | | | | 1936 | | | |
| Number and Location | Feed | Number | Litter Number | Sex | Weight at Start | 6/14 | 6/29 | 7/30 | 8/31 | 10/2 | 11/1 | 12/3 | 12/31 | 1/30 | 2/29 | 3/28 | 5/2 | 6/6 |
| 3 Outside | Ready-mixed feed | 401 | 6 | M | | .26+ | .24 | .24 | .22 | .20 | .10 | .10 | .08 | .08 | .10 | .10 | .08 | .12 |
| | | | | | 80 | 108 | 131 | 256 | 421 | 500 | 602 | 620 | 598 | 604 | 701 | 663 | 643 | 738 |
| | Cabbage | 316 | 11 | M | | | | | .20 | .16 | .14 | .10 | .08 | .08 | .08 | .08 | .06 | .06 |
| | | | | | 73 | | | | 160 | 336 | 468 | 540 | 530 | 550 | 655 | 650 | 627 | 649 |
| | | 183 | 3 | F | | | .24 | .22 | .24 | .24 | .18 | .20 | .20 | .20 | .24 | .22 | .26+ | .12 |
| | | | | | 78 | 94 | 113 | 230 | 406 | 520 | 625 | 684 | 809 | 616 | 980 | 746 | 1010 | 753 |
| | | 422 | 8 | F | | .26+ | .24 | .12 | .14 | .12 | .16 | .26 | .12 | .22 | .22 | .12 | .24 | .20 |
| | | | | | 92 | 122 | 165 | 287 | 436 | 525 | 631 | 796 | 570 | 602 | 877 | 714 | 884 | 796 |
| | | 181 | 2 | F | | .26+* | | | | | | | | | | | | |
| | | | | | 102 | 121 | | | | | | | | | | | | |
| 3 Inside | Ready-mixed feed Cabbage | 415 | 6 | M | | .24 | .20 | .10 | .10 | .12 | .14 | .10 | .10 | .08 | .10 | .10 | .10 | .08 |
| | | | | | 77 | 114 | 167 | 358 | 520 | 634 | 684 | 676 | 698 | 725 | 778 | 796 | 800 | 850 |
| | | 184 | 3 | F | | .24 | .20 | .22 | .24 | .24 | .26+ | .18 | .18 | .18 | .20 | .22 | .24 | 126 |
| | | | | | 136 | 184 | 254 | 396 | 504 | 745 | 656 | 680 | 815 | 768 | 977 | 1382 | 856 | 800 |
| | | 190 | 5 | F | | .26+ | .26+ | .12 | .22 | .24 | .24 | .26 | .26+ | .22 | .20 | .22 | .26+ | .20 |
| | | | | | 193 | 247 | 300 | 415 | 560 | 822 | 744 | 881 | 712 | 894 | 936 | 886 | 1434 | 925 |
| | | 199 | 9 | F | | * | | | | | | | | | | | | |
| | | | | | 202 | | | | | | | | | | | | | |

* Died

** Weight expressed in grams.

Note: Large increases in weight attended by subsequent fall in weight in females was due to pregnancy.

Cages and Distribution: Three cages were placed outdoors to determine what effect sunlight and various temperatures might have on the titer of complement. Animals in these cages were allowed to remain outside regardless of weather conditions, except from January 23 to February 26, 1936, when unusually heavy and continued snowfall made it necessary to move them to an unheated barn.

Three cages were kept indoors in a steam-heated animal house, especially constructed to accommodate small laboratory animals, and where the temperature could be regulated throughout the year.

The animals were placed in rectangular cages 3' x 1½' x 1' deep and set on legs 1½' high. The sides of the cages were covered with fine mesh wire nailed to a wooden frame. The top, covered with the same type of wire, was hinged so that it could be raised. The floor was constructed of wood over which a heavy, removable, galvanized iron tray was placed. In the outdoor cages one-half of each cage was covered, including half the top, half the sides, and the corresponding end, with heavy tar roofing paper to afford protection from the weather. Under the covered end was placed a small wooden box, one end of which had an opening in order to give the animals additional protection. The other half of the cage remained open for the admission of sunlight.

The animals on test were placed in the various cages, as shown in Table X, so as to give the best possible distribution of litter mates. This distribution was made in order to give, as far as possible, a comparison of litter mates reared under the various conditions of the experiments.

## TABLE X

### A Comparison of Complement Titers, Age, and Sex of Guinea Pig Litter Mates

(Results of titration expressed as cc. of 1:10 complement required to hemolyze 0.5 cc. sheep cells with 2 units of hemolysin (0.5 cc. of 1:2,000 dilution).)

| Guinea Pig | | | | Date of Determining Titer | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Litter | | | Cage Number | 1935 | | | | | | | | 1936 | | | | |
| Number | Sex | Number | and Location | 6/14 | 6/29 | 7/30 | 8/31 | 10/2 | 11/1 | 12/3 | 12/31 | 1/30 | 2/29 | 3/28 | 5/2 | 6/6 |
| 1 | M | 409 | 1, outside | .18 | .24 | .22 | .18 | .22 | .18 | .10 | .10 | .12 | .10 | .08 | .12 | .14 |
| | M | 177 | 1, inside | .22 | .24 | .22 | .20 | .16* | | | | | | | | |
| | F | 178 | 2, outside | .26+ | .26+ | .18 | .24 | .12 | .16 | .16 | .20 | .12 | .26 | .24 | .20 | .26+ |
| 2 | M | 418 | 2, outside | .20 | .20 | .18 | .20 | .20 | .18 | .10 | .10 | .08 | .10 | .10 | .12 | .14 |
| | F | 180 | 2, inside | .22 | .20 | .12 | .14 | .14 | .24 | .12* | | | | | | |
| | F | 181 | 3, outside | .26+* | | | | | | | | | | | | |
| 3 | F | 410 | 1, outside | .26 | - | .22 | .16 | .16 | .20 | .14 | .16 | .24 | .18 | .26 | .26+ | .26 |
| | F | 183 | 3, outside | - | .24 | .22 | .24 | .24 | .18 | .20 | .20 | .20 | .24 | .22 | .26+ | .12 |
| | F | 184 | 3, inside | .24 | .20 | .22 | .24 | .24 | .26+ | .18 | .18 | .18 | .20 | .22 | .24 | .26 |
| 4 | M | 186 | 2, inside | .20* | | | | | | | | | | | | |
| | F | 406 | 1, inside | .16 | .18 | .16 | .18 | .26+ | .20 | .20 | .26 | .20 | .14 | .12 | .10 | .20 |
| | F | 187 | 1, outside | .16 | .16 | .20 | .20 | .22 | .18 | .22 | .12 | .22 | .12 | .26 | .18 | .24 |
| 5 | M | 189 | 2, inside | .24 | .20 | .18 | .18 | .22 | .16 | .16 | .22 | .10 | .10 | .08 | .08 | .12 |
| | F | 403 | 2, outside | .20 | .16 | .20 | .20 | .20 | .12 | .12 | .22 | .10 | .20 | .22 | .24 | .26 |
| | F | 190 | 3, inside | .26+ | .26+ | .12 | .22 | .24 | .24 | .26 | .26+ | .22 | .20 | .22 | .26+ | .20 |
| 6 | M | 415 | 3, inside | .24 | .20 | .10 | .10 | .12 | .14 | .10 | .10 | .08 | .10 | .10 | .10 | .08 |
| | M | 401 | 3, outside | .26+ | .24 | .24 | .22 | .20 | .10 | .10 | .08 | .08 | .10 | .10 | .08 | .12 |
| | F | 191 | 1, inside | .24 | .24 | .22 | .20 | .22 | .14 | .12 | .16 | .26+ | .12 | .12 | .18 | .14 |

TABLE X
(continued)

| Guinea Pig | | | | Date of Determining Titer | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Litter | | | Cage Number | 1935 | | | | | | | | 1936 | | | | |
| Number | Sex | Number | and Location | 6/14 | 6/29 | 7/30 | 8/31 | 10/2 | 11/1 | 12/3 | 12/31 | 1/30 | 2/29 | 3/28 | 5/2 | 6/6 |
| 7 | M | 194 | 1, inside | .22 | .20 | .22 | .22 | .24 | .16 | .16* | | | | | | |
| | M | 195 | 2, inside | .20 | .24 | .20 | .24 | .24 | .16 | .16 | .14 | .10 | .10 | .10 | .14 | .16 |
| 8 | M | 413 | 2, outside | .22 | .22 | .20 | .20 | .12 | .16 | .14 | .10 | .14 | .12 | .12 | .12 | .10 |
| | F | 422 | 3, outside | .26+ | .24 | .12 | .14 | .12 | .16 | .26 | .12 | .22 | .22 | .12 | .24 | .20 |
| 9 | M | 198 | 1, outside | .18 | .18 | .22 | .24 | .20 | .16 | .10 | .10 | .10 | .08 | .08 | .10 | .10 |
| | F | 199 | 3, inside | * | | | | | | | | | | | | |
| 10** | M | 408 | 1, outside | | | | .26+ | .26+ | .20 | .18 | .10 | .12 | .10 | .12 | .10 | .10 |
| 11** | M | 316 | 3, outside | | | | .20 | .16 | .14 | .10 | .08 | .08 | .08 | .08 | .06 | .06 |
| 12** | M | 420 | 2, inside | | | | .24 | .20 | .16 | .14 | .12 | .12 | .12 | .10 | .12 | .10 |
| | M | 301 | 1, inside | | | | .26+ | .26+ | .18 | .10 | .08* | | | | | |

* Died

** Litters 10, 11, and 12 were added August 31, 1935 to replace animals that died.

Rations: The guinea pigs placed in the six cages, three outdoors and three indoors, were fed one of the following three different rations commonly used. Fresh water was available at all times.

Ration 1 consisted of a grain mixture of wheat and oats, with greens fed each day in the form of cabbage, kale, lettuce, grass, or spinach. Ration 2 contained oats and a pellet form of food consisting of wheat germ, soy bean oil meal, corn germ meal, alfalfa leaf meal, ground oats, wheat middlings, yellow corn meal, blackstrap molasses, calcium carbonate, and iodized salt. Cabbage was fed daily as a source of vitamin C. Ration 3 was a commercially prepared, ready-mixed product, consisting of chopped alfalfa, crushed oats, yellow cracked corn, blackstrap molasses, and a pellet feed composed of wheat germ meal, soy bean oil meal, corn germ meal, wheat middlings, calcium carbonate, and iodized salt. Cabbage was fed with this ration on alternate days.

The guinea pigs were fed and watered in the early morning once a day. The cages were cleaned and bedded with straw twice a week. When necessary the floors were allowed to dry before the straw was added. Weights were recorded each week but tabulated only at the time that each titration was made.

RESULTS: Data are available on twenty-five of the twenty-eight animals used during this work. Four of the fifteen males died before the completion of the experiment and no data could be collected on one of these. Three of the thirteen females used died and no information could be obtained on two of these.

In all cages, regardless of diet, the blood serum of the male guinea pigs showed a gradual increase in titer starting between the third

and sixth month. Of the animals surviving until the completion of the test period, however, five showed some slight drop in titer during the last month or two (May and June), and the titer of six remained about equal to the highest titer during the entire experiment. Three of the animals which died before the completion of the experiment showed an increased titer. Considerable individual variation has often been reported previously in mature animals, and as shown in Table IX, the weights of these animals could not be correlated with the slight drop in complement strength. Table X shows the five animals distributed in four different cages. Whether the cages were indoors or outdoors is probably not significant, although the animals in two of the three inside cages did not show a titer drop. However, in one of these (cage 1) the three animals did not live until the completion of the experiment.

The females almost invariably showed a considerably lower titer than males, beginning between the fourth and sixth month, although their original complement content might have been equal or better. An exception occurred in one cage (cage 1 inside) where all male animals died at least four months before the experiment was completed. One other animal (cage 3 outside) did give a decidedly higher titer of 0.12 cc. at the last bleeding. This usual loss of hemolytic activity in females was probably due to the interference of pregnancy, a factor which has been discounted by certain investigators. An examination of Table IX shows this lowering of complement content during pregnancy. Weekly weights show a larger increase and decrease in weight, due to pregnancy and parturition, than the monthly weights shown in Table IX. Because females will almost invariably breed as soon as the young are delivered, after the first sev-

eral months, they were nearly always in a state of pregnancy since the cages always contained a mixture of males and females, with one exception (cage 1 inside). The titers of the females, while nearly always poor during pregnancy, were lowest near the end of their terms. Frequently when the weights as shown in Table IX would indicate a litter had just been born, and the female had not begun to show signs of another pregnancy, the titers were considerably higher.

Because of this interference of pregnancy with complement titer, few, if any, conclusions could be drawn with regard to the influence of feeding, sunlight, temperature, weight, or litter mate comparisons of females. At some institutions, where fresh complement is used in rather large amounts, old female guinea pigs that are no longer breeding are preferred. While a check on such animals was beyond the scope of this work, no significant differences were observed in titer between young males and females ranging in weight between 400 and 800 gms., if the females observed to be pregnant were eliminated.

Guinea pigs reach maturity in from four to five months according to a report of the United States Department of Agriculture (59), but, as seen in Table IX, the maximum weight was not reached at that time. Whenever the titer of the serum of male animals increased to 0.16 cc. in these studies, they had also reached a weight of 450 gms. or more (Table IX). Three animals, No. 301 (cage 1 inside), No. 316 (cage 3 outside), and No. 415 (cage 3 inside), were exceptions. These animals all belonged to different litters and weighed between 336 and 376 gms. All were in different cages. Two of them were raised inside and two were fed the same diet. This is probably not significant enough to warrant any conclusions.

A titer of 0.1 cc. was not obtained until a weight of better than 525 gms. had been reached by all pigs with the exception of two of the three mentioned above (No. 301, cage 1 inside, and No. 415, cage 3 inside). Table IX shows that seven animals weighed 600 gms. or more before the titer of their complement reached 0.1 cc.

Young male animals did not show a titer of 0.14 cc. or better until they had reached an approximate age of four months or more. The majority of animals were between five and seven months of age before the titer of 0.1 cc. was reached. Animals No. 316 (cage 3 outside) and No. 415 (cage 3 inside) were exceptions, showing a titer equivalent to or better than this in three and two months, respectively. As difficult as it was to get data on female guinea pigs, what has been indicated about the males, in general, holds true for females.

In considering female litter mates raised in different cages, it is difficult to obtain significant results, largely because of the interference of pregnancy with complement activity. Additional complications arose where litters contained both males and females. However, some information may be obtained by a comparison of litters 3 and 4 in Table X. All of these animals were females except one male in litter 4 that died after the first bleeding. Two guinea pigs in litter 4 had a higher initial titer than those of litter 3 and tended to maintain it throughout the duration of the experiment. One animal in each litter was raised in cage 1 inside (No. 410, litter 3 and No. 187, litter 4) where identical conditions of maintenance did not seem to equalize their titers. While, at the beginning, the complement titers of all the animals were low, variations among the animals of the same litters were noticeable. This is shown in litter 5 where No. 403 had a higher titer than No. 190 for nine bleedings,

twice had a titer equal to No. 190 and in two other instances the titers were lower than No. 190. Both pigs were females. In comparing female and male litter mates, the males invariably reached and maintained a higher titer, although this comparison may be unfair because of pregnancy. One animal, a female of litter 6 (No. 191, cage 1 inside) lived at least the last four months without being pregnant and her titer never equalled that of the two males of the same litter.

Only in litter 6 did the two male litter mates live to the completion of these studies, and, while there were differences in titer up to the end of the sixth month, their complement strengths were nearly parallel throughout the balance of the experiment. In litter 7, where seven comparable titers of two males were obtained, the titers again paralleled one another. All members of both of these litters (6 and 7) were raised under different conditions (Table X).

Definite conclusions regarding male litter mate comparisons can hardly be reached because the number of comparisons were too few. It should be noted that all of the animals came from good stock, and that all of the male guinea pigs, by the time they had been on test eight months or less, had reached a titer of 0.1 cc. Males of some litters reached that strength complement before males of other litters. For example, No. 198, litter 5, required eight months, whereas No. 316, litter 11, required only half that time.

In preparing the three diets, no attempts were made to feed guinea pigs deficient food but rather to use rations that have been successfully used in various laboratories where pigs are raised as a source of complement.

Again data on the females in the various cages are uncertain for the reasons discussed above. As indicated in Table IX, cage 1 outside, titers as great as 0.12 cc. were attained in only two instances, and those were in the same pig (No. 187). In only one instance did this titer appear in females given the same ration but housed inside during the time they were with males. This is possibly due to the lack of maturity, as pointed out in discussion of titers and age. In cage 2 outside, the two females, No. 178 and No. 403, had two titers each of 0.12 cc. and No. 403 showed a titer of 0.10 cc. in one instance, at a time when, as is shown by Table IX, she had just previously given birth to young. The one female in cage 2 inside had a fairly good titer for a young animal from the end of the second month on until death, except at the time when she was obviously heavy with pigs. Guinea pig No. 422, in cage 3 outside, on four occasions had a titer of 0.12 cc., and No. 183, a cage mate, only once achieved an equal complement strength. Cage 3 inside contained, from the standpoint of breeding, two exceptionally fine females. Both had as many as five pigs in a litter and raised them all, yet only once was a satisfactory titer reached (No. 190). Little difference was apparent between animals housed outdoors and exposed to sunlight and widely fluctuating temperatures, and those kept indoors away from direct sunlight at rather uniform temperatures throughout the year.

The young were allowed to stay with the mother four weeks before removal to separate pens. The effect of nursing young upon the titer of complement was not investigated.

Two of the males in cages 3 indoors and out (guinea pigs No. 410 and No. 401 respectively, both of litter 6) had sera of high complement

content. Another male (No. 316, cage 3 outside) also had a high titer serum. All three reached a titer of 0.10 cc. in at least five months. Males in cage 2 took longer to reach titers equivalent to those in cages 3. Those indoors required eight months, and the two outdoors required six and seven months. The three males in cage 1 inside all died before the experiment was completed. Only one, No. 301, had a titer of 0.10 cc., this figure being reached in four months. Sera of the others failed to reach this strength. The cage 1 outside housed two males and both acquired a titer of 0.10 cc. by the sixth month.

Sunlight and temperature apparently did not have any appreciable effect on complement titer; although the animals in cage 2 inside required a little longer time to reach a satisfactory titer than the rest. Cage 3 inside, with its one male, had the first of all guinea pigs to reach a titer of 0.1 cc. The animals in cages 1 and 2 outside were about equal, and those in cage 3 outside reached a satisfactory titer a little in advance of those in the other outside cages.

From the information assembled in Table IX, the ration fed males of cages 3 showed some slight superiority over the other two, but it must be borne in mind that these cages housed males of litter 6, a superior pair of guinea pigs. No. 316, fed on the same ration, had no other mate for comparison. However, we would favor this ration because it is well balanced, easily handled, reasonably cheap, and necessitates green feed only every other day, and because all males on this diet showed a high titer in a reasonable time.

During the year's study, the animals housed outdoors were kept there regardless of the weather. As young animals they did very well

through the hot summer months when it was not uncommon for the temperature to reach 100° F. Usually, however, they preferred the shade and thus it is difficult to compare this study with that of Remlinger and Bailly (45) who found that rodents died if left in the sun for as short a period as a half hour. During December 1935, and January and February 1936, the temperature was usually near freezing and subzero weather was not uncommon with unusually heavy snowfall. For this entire period the guinea pigs were kept outdoors except from January 23 to February 26, when they were housed in an unheated barn. Examination of the titers recorded in Table IX would not indicate that this extreme weather had any lowering effect upon the strength of complement. They were always equal to and in some instances better than in the corresponding cages inside. The pigs housed indoors were kept at a rather uniform temperature throughout the year, conforming to the recommendations of the United States Department of Agriculture (59).

Between July 6 and 10, 1936, after the work was completed, it was interesting to note that the temperature reached 104° F. with high humidity prevalent. Six of the animals of the outdoor group died. They had been allowed to stay outside and were treated just as though on experiment. Post-mortem examinations revealed only congested lungs, probably due to rapid respiration, indicating that the pigs had died of suffocation probably as a result of the intense heat and high humidity. Had the pens been larger with more space for the circulation of air, this might not have happened. A year before the young animals were exposed to heat as extreme as that encountered in 1936, but were apparently able to withstand the excessive heat better than the mature animals. During the period of this extremely

hot weather, not a single death occurred among the animals in the inside cages.

While it is not recommended as a result of this work that animals be reared outdoors, it is noteworthy that the changes of temperature had no effect upon complement strength. The animals did survive extreme cold very well, and with larger and better aerated pens, sheltered from the sun, could probably have survived the excessive heat.

## C. A Comparison of Methods for the Preservation of the Hemolytic Activity of Guinea Pig Complement

Three or more guinea pigs are usually bled to obtain a uniformly satisfactory complement. Often some of this fresh complement is left over and unless special methods for preservation are used the serum rapidly deteriorates. Suitable methods of preservation are also valuable as they eliminate the necessity of bleeding animals each time complement is needed and further allow for the utilization of guinea pigs for serum at times when large numbers are on hand. Since we have investigated some methods for measuring and producing guinea pig serum of a satisfactory titer, it seemed desirable to study some of the more widely used methods for the preservation of this titer.

Preparation of Materials: Complement was obtained from healthy male or non-breeding female guinea pigs, all one year old or more. The sera of from six to fifteen animals were pooled to make up the sample to be tested. The procedure for obtaining and preparing corpuscles and complement sera was carried out as described under "Preparation of Materials" A. 1.

The sample of Lyo Complement* prepared by Sharp and Dohme after the method of Flosdorf and Mudd was reported to have been prepared from the pooled sera of fifty males.

For determining the hemolytic titer of preserved complements, the same antisheep hemolysin was employed throughout the experiment. Two units contained in 0.5 cc. of a 1:3,000 dilution were used. Frequent titrations

*Printed circular LR-44 accompanying packaged serum. Mulford Biological Laboratories, Sharp & Dohme, Philadelphia, Pa.

showed that no deterioration had taken place.

Saline was prepared by dissolving 8.5 gms. C.P. sodium chloride and 0.1 gm. magnesium chloride in cold tap water, making up to one liter in volume, and autoclaving at fifteen pounds' pressure for thirty minutes.

All guinea pigs used for this experiment were fed a commercially prepared feed that is described as ration 3 under B. They were all housed in the animal building. The feed was removed eighteen to twenty-four hours before bleeding.

The various samples of complement, in three-cc. portions, were stored in sterile, rubber-stoppered, glass vials with the exception of the samples preserved with carbon dioxide which were plugged with cotton. Samples of one cc. were used for the freezing experiments. The time consumed for removal from the refrigerator, sampling, and returning was approximately two minutes for refrigerated samples.

Complement Titration: The complement was titrated by determining the least amount necessary to bring about complete hemolysis of 0.5 cc. of a 2 per cent suspension of sheep cells in the presence of two units of hemolysin. The complements were diluted 1:10, 1:20, or 1:30 as was found necessary according to the method of preservation. When a 1:10 dilution was necessary, varying amounts ranging from 0.06 cc. to 0.30 cc. with a 0.02-cc. interval were used. If a 1:20 dilution was needed, varying amounts ranging from 0.1 cc. to 0.6 cc. with a 0.05-cc. interval were employed. Dilutions of 1:30 ranged from 0.1 cc. to 0.9 cc. with a 0.05-cc. interval. The total volume in all cases was made up to 3 cc. with saline. Saline, hemolysin, and complement controls were set up for each titration. In all experiments the results of the complement titrations were read after one hour's incubation in a water bath at 37° C.

Temperatures: All room temperatures recorded ranged between 21° and 26° C. All electrical refrigerator temperatures ranged between 7° and 9° C. except when the freezing compartment was used and then the temperature was approximately -8° C.

Methods of Preservation: Controls - Three samples of complement, in duplicate, were kept, without the addition of any preservative. One set of three was held at room temperature and the others in an electric refrigerator. Dilutions of 1:10 were prepared for titration by adding nine volumes of saline to one of complement.

Carbon dioxide - Three samples of complement, two of which were in duplicate, were preserved by displacing the oxygen in the containers with carbon dioxide as follows: The sample was placed in a Novy jar, the top of which had two openings. The jar was sealed with a vaselined rubber ring and clamped shut. Carbon dioxide from a commercial tank was run in through one opening in the top of the jar and the oxygen was forced out through the other opening. After approximately three minutes, when practically all the oxygen was displaced, the openings were closed with screw clamps. Two sets were maintained at room and electric refrigerator temperatures while the third was kept only in the electric refrigerator. Dilutions of 1:10 were prepared for titration by adding nine volumes of saline to one of preserved serum.

Sodium acetate - A 12 per cent solution of sodium acetate in 0.85 per cent solution of sodium chloride was prepared and sterilized. Duplicate samples were prepared by mixing four parts of complement and six parts of the acetate solution.* One sample was stored at room temperature

*The mixture contained approximately 8 per cent sodium acetate.

and the other in the electric refrigerator. A 1:20 dilution was made up for titration by adding one part of the preserved complement to seven parts of distilled water.

Sodium acetate with powdered or crystalline boric acid - Two samples were prepared in duplicate, one by adding 0.1 gm. of sodium acetate and 0.04 gm. of powdered boric acid to each cc. of serum; the other, by substituting 0.03 gm. of crystalline boric acid for 0.04 gm. of powdered boric acid. This was done because some precipitate was noticeable in the first sample, and the crystalline boric acid went into solution much more readily. One of each of the duplicates was held at room temperature and the others in an electric refrigerator. A 1:10 dilution for titration was prepared by adding nine volumes of distilled water to one volume of preserved serum.

Sodium chloride - A saturated solution of C.P. sodium chloride was sterilized. Duplicate samples were prepared by adding 0.1 cc. of the salt solution to each cc. of serum.* One of the duplicates was held at room temperature and the other in the electric refrigerator. For titration, a dilution of 1:10 was made by the addition of three volumes of distilled water and six of saline to one of preserved complement.

Sodium chloride (17 per cent) - Duplicate samples were prepared by dissolving 0.17 gm. of sodium chloride in each cc. of serum. One of the duplicates was held at room temperature and the other in the electric refrigerator. A dilution of 1:20 was made up for titration by adding nineteen volumes of distilled water to one volume of preserved complement.

Sodium chloride (25 per cent) - Duplicate samples were prepared by dissolving 0.25 gm. of sodium chloride in each cc. of serum. One of the duplicates was held at room temperature and the other in the electric refrigerator. A 1:30 dilution was obtained for titration by adding twenty-

* The mixture contained about 3 per cent sodium chloride.

nine volumes of distilled water to one volume of the preserved complement.

Sodium chloride with powdered or crystalline boric acid (10 per cent) - Preparation and treatment of samples, and final dilution were done as with sodium acetate with powdered or crystalline boric acid.

Sodium chlorate and crystalline boric acid - Two samples of complement were prepared in duplicate by adding 0.1 gm. of sodium chlorate and 0.04 gm. crystalline boric acid to each cc. of serum. Two of the duplicates were held at room temperature and the others in the electric refrigerator. The preserved complement was diluted 1:10 for titration by adding nine volumes of distilled water to one volume of preserved serum.

Sodium fluoride - Duplicate samples were made up by mixing 0.04 gm. of sodium fluoride with each cc. of serum. One sample was held at room temperature and the other in the electric refrigerator. A dilution of 1:10 for titration was prepared by adding four volumes of distilled water and five of saline to one of preserved complement.

Strontium chloride with crystalline boric acid - Two samples were prepared in duplicate by adding 0.05 gm. of strontium chloride and 0.04 gm. crystalline boric acid to each cc. of serum. Two of the duplicates were held at room temperature and the others in the electric refrigerator. A dilution of 1:10 was prepared for titration by adding five volumes of distilled water and four of saline to one of preserved serum.

Magnesium sulfate - Duplicate samples were prepared by adding 0.05 gm. magnesium sulfate to each cc. of serum. One sample was held at room temperature and the other in the electric refrigerator. A dilution of 1:10 was prepared for titration by adding five volumes of distilled water and four of saline to one of preserved serum.

Freezing - Six one-cc. samples were placed in the freezing compartment of an electric refrigerator at a temperature of approximately -8° C. A dilution of 1:10 was prepared with saline after the sample was

thawed. The remainder of the sample was discarded as a new sample was used for each titration.

Dehydration - Eight one-cc. samples were prepared by the freezing-dehydration method of Flosdorf and Mudd (13). The dehydration was carried out for three hours. Four samples were held at room temperature and the remaining four in the electric refrigerator. Two additional three-cc. samples were dehydrated for twenty-two hours and stored in the refrigerator. A five-cc. sample of Lyo Complement (Sharp and Dohme) was also used. All samples were restored to their original volumes by the addition of distilled water, and diluted 1:10 for titration by adding nine volumes of saline to one of restored complement.

Dehydration and salting - Two three-cc. samples of complement were salted and dehydrated twenty-two hours by the same method as above. To sample 1 was added 0.15 cc. and to No. 2 was added 0.30 cc. of a 17 per cent salt solution. They were stored in the electric refrigerator. Both were restored to isotonicity, the former receiving six cc. of distilled water and the latter nine cc. They were further diluted to 1:10 with saline and titrated.

Freezing restored-dehydrated complement - A portion of the Lyo Complement which was restored to its original volume but undiluted, was stored in one-cc. samples at -8° C. in the freezing compartment of the refrigerator. A 1:10 dilution for titration was prepared by adding nine volumes of saline to one of thawed complement.

Sodium chloride with crystalline boric acid, restored-dehydrated complement - Another portion of the Lyo Complement was preserved by adding 0.1 gm. sodium chloride and 0.03 gm. of crystalline boric acid to

each cc. of restored serum and then refrigerating. A 1:10 dilution for titration was prepared by adding nine volumes of distilled water to one volume of preserved serum.

Carbon dioxide, restored-dehydrated salted complement - The remainder of the restored sample of the twenty-two-hour dehydrated sample to which 0.15 cc. of 17 per cent salt solution per cc. had been added was preserved by replacing the oxygen in a Novy jar with carbon dioxide as explained above. The sample was stored in the electric refrigerator. A 1:10 dilution for titration was prepared by adding four volumes of saline to one of preserved complement.

Sodium chloride, restored-dehydrated unsalted complement - Each cc. of restored complement was preserved by adding 0.17 gm. of sodium chloride. The sample was stored in the electric refrigerator. A 1:20 dilution for titration was prepared by adding nineteen volumes of distilled water to one volume of preserved complement.

RESULTS: In determining the effect of various methods of complement preservation on hemolytic titer, 0.30 cc. of a 1:10 dilution, or its equivalent, was considered as the end point or titer beyond which complement would be valueless in complement fixation reactions. All titration figures given refer to the use of complement in dilutions of 1:10. Tables XI, XII, XIII, and XIV contain the results obtained with the various methods of preservation, including the time at which the end points were reached, or at which the samples were depleted or contaminated.

TABLE XI

Hemolytic Titer of Unpreserved Complement

(Results expressed in cc. of a 1:10 dilution)

| Test No. | Titer when Fresh | Storage | | | | |
|---|---|---|---|---|---|---|
| | | | Duration - Days | | | |
| | | Type | 3 | 5 | 10 | 12 |
| I | .04 | Refrig. | - | .18 | - | .30+ |
| | | Room | - | .30+ | - | - |
| II | .06 | Refrig. | .18 | - | .30+ | - |
| | | Room | .30+ | - | - | - |
| III | .08 | Refrig. | .14 | - | .30+ | - |
| | | Room | .30+ | - | - | - |

Unpreserved complement: Unpreserved complement held at room temperature reached a titer greater than 0.30 cc. between the third and fifth days. This titer may have been reached earlier, but the above-mentioned times were the first at which a titer was determined. The drop was not so rapid when the serum was held in the refrigerator, a titer greater than 0.30 cc. appearing between the tenth and twelfth days. This titer probably was reached sooner, but an interval of one week was allowed between the first and second titrations. Results are indicated in Table XI.

Carbon dioxide: Refrigerator temperature was superior in preservative action to room temperature. The titers of both samples kept at room temperature had decreased more than half that of the same samples when fresh at the end of the first week. The first sample treated and kept at refrigerator temperature maintained its original titer for three weeks. At the end of eight weeks the titer dropped only 0.02 cc., and when the same sample was depleted at the end of thirteen weeks the titer had dropped only 0.06 cc. when compared to the fresh sample. The other two samples,

## TABLE XII

### Hemolytic Titer of Complement Preserved by Various Methods

(Results expressed in cc. of a 1:10 dilution)

| Treatment of sample | % | Titer when Fresh | Storage Type | Duration - Weeks 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Carbon dioxide | | .08 | Refrig. | .08 | .08 | .08 | .10 | .10 | .10 | .10 | .10 | .12 | .14 | .14 | .14 | .14 | sample depleted | | | |
| | | | Room | .18 | .18 | .18 | .18 | .18 | .18 | .30+ | | | | | | | | | | |
| Carbon dioxide | | .08 | Refrig. | .14 | .14 | .14 | .16 | .18 | .18 | .20 | .20 | .20 | .20 | .20 | .20 | .20 | .20 | .20 | .20 | .20 depleted |
| | | | Room | .26 | .26 | .30+ | | | | | | | | | | | | | | |
| Carbon dioxide | | .10 | Refrig. | .14 | .14 | .16 | .20 | .20 | .20 | .20 | .20 | .22 | .24 | .24 | .24 | .24 | .30+ | | | |
| Sodium acetate* | 12 | .06 | Refrig. | .08 | .08 | .10 | .10 | .13 | .13 | .13 | .13 | .13 | .18 | .18 | .18 | .20 | .20 | .30+ | | |
| | | | Room | .18 | .18 | .20 | .23 | .30+ | | | | | | | | | | | | |
| Sodium acetate, Boric acid | 10 4 | .06 | Refrig. | .06 | .06 | .06 | .10 | .10 | .12 | .18 | .20 | .22 | .22 | .26 | .30+ | | | | | |
| | | | Room | .06 | .08 | .08 | .18 | .18 | .30+ | | | | | | | | | | | |
| Sodium acetate, Boric acid | 10 3 | .10 | Refrig. | .12 | .12 | .12 | .12 | .14 | .16 | .16 | .16 | .16 | .16 | .16 | .22 | .30+ | | | | |
| | | | Room | .14 | .14 | .14 | .14 | .14 | .18 | .30+ | | | | | | | | | | |
| Sodium chloride | Sat. | .06 | Refrig. | .06 | .06 | .10 | .12 | .12 | .14 | .26 | .26 | .30+ | | | | | | | | |
| | | | Room | .06 | .14 | .30+ | | | | | | | | | | | | | | |
| Sodium chloride* | 17 | .04 | Refrig. | .05 | .10 | .10 | .10 | .10 | .10 | .13 | .13 | .13 | .13 | .13 | .13 | .13 | .18 | .23 | .25 | .30+ |
| | | | Room | .05 | .10 | .15 | .20 | .23 | .30+ | | | | | | | | | | | |
| Sodium chloride** | 25 | .06 | Refrig. | .07 | .07 | .10 | .12 | .14 | .14 | .15 | .19 | .19 | .19 | .19 | .19 | .20 | .20 | .22 | .25 | .27*** |
| | | | Room | .10 | .10 | .15 | .20 | .30+ | | | | | | | | | | | | |

TABLE XII
(continued)

| Treatment of sample | % | Titer when Fresh | Type | Storage Duration - Weeks 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sodium chloride | 10 | .06 | Refrig. | .06 | .06 | .12 | .16 | .16 | .16 | .16 | .22 | .22 | .22 | .22 | .22 | .24 | .30+ | | | |
| Boric acid | 4 | | Room | .26 | .30+ | | | | | | | | | | | | | | | |
| Sodium chloride, | 10 | .10 | Refrig. | .12 | .12 | .12 | .12 | .12 | .12 | .16 | .16 | .16 | .16 | .16 | .20 | contaminated | | | | |
| Boric acid | 3 | | Room | .12 | .12 | .12 | .12 | .12 | .16 | .30+ | | | | | | | | | | |
| Sodium chlorate, | 10 | .06 | Refrig. | .30+ | | | | | | | | | | | | | | | | |
| Boric acid | 4 | | Room | .30+ | | | | | | | | | | | | | | | | |
| Sodium chlorate, | 10 | .10 | Refrig. | .30+ | | | | | | | | | | | | | | | | |
| Boric acid | 4 | | Room | .30+ | | | | | | | | | | | | | | | | |
| Sodium fluoride | 4 | .08 | Refrig. | .08 | .08 | .10 | .12 | .14 | .14 | .18 | .18 | .26 | .30+ | | | | | | | |
| | | | Room | .30+ | | | | | | | | | | | | | | | | |
| Strontium chloride, | 5 | .08 | Refrig. | .30+ | | | | | | | | | | | | | | | | |
| Boric acid | 4 | | Room | .30+ | | | | | | | | | | | | | | | | |
| Strontium chloride, | 5 | .10 | Refrig. | .30+ | | | | | | | | | | | | | | | | |
| Boric acid | 4 | | Room | .30+ | | | | | | | | | | | | | | | | |
| Magnesium sulfate | 5 | .10 | Refrig. | .12 | .20 | .24 | .30+ | | | | | | | | | | | | | |
| | | | Room | .24 | .30+ | | | | | | | | | | | | | | | |
| Freezing | | .10 | -8° C. | .12 | .24 | .28 | .30+ | | | | | | | | | | | | | |

*Titrations made in dilutions of 1:20. Results converted to 1:10.
** Titrations made in dilutions of 1:30. Results converted to 1:10.
*** Titer of 0.27 cc. maintained through nineteenth week; titer of 0.30+ reached during twentieth week.

TABLE XIII

Hemolytic Titer of Dehydrated Complement

(Results expressed in cc. of a 1:10 dilution)

| Treatment of Sample | Titer when Fresh | Storage | | | | |
|---|---|---|---|---|---|---|
| | | | Duration - Months | | | |
| | | Type | 1 | 2 | 3 | 4 |
| Dehydrated 3 hours | .06 | Refrig. | .26 | .30+ | | |
| | | Room | .30+ | | | |
| Dehydrated 22 hours* | | | | | | |
| Sample 1 | .10 | Refrig. | .10 | | | |
| Sample 2 | .10 | Refrig. | | | | .10 |
| Dehydrated 22 hours** | .10 | Refrig. | | .10 | | |
| Dehydrated 22 hours** | .10 | Refrig. | | | | .10 |
| Lyo Complement (restored) | .12 | Refrig. | Age and titer of original unknown. | | | |

* Unsalted

** Salted with .15 cc. and .30 cc. of 17 per cent sodium chloride respectively before dehydration.

TABLE XIV

Hemolytic Titer of Restored Dehydrated Complement, Further Preserved by Various Methods

(Results expressed in cc. of a 1:10 dilution)

| Source | Treatment | % | Titer when Restored | Storage | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Duration - Weeks | | | | | | | | |
| | | | | Type | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Lyo Complement | Freezing | | .12 | -8°C. | .26 | .26 | .30+ | | | | | | | |
| Lyo Complement | Sodium chloride Boric acid | 10 3 | .12 | Refrig. | .20 | .22 | .22 | .24 | .24 | .24 | .24 | .24 | .24 | 30+ |
| Dehydrated 22 hrs. (salted) | Carbon dioxide | | .10 | Refrig. | .20 | .20 | .20 | .20 | .20 | .20 | .22 | .30+ | | |
| Dehydrated 22 hrs. (unsalted) | Sodium chloride | 17* | .10 | Refrig. | .15 | .15 | .18 | .20 | .20 | .20 | .20 | .20 | .20 | 24* |

* Seventeen per cent sodium chloride was titrated in a dilution of 1:20. Results were converted to 1:10.

** Depleted.

while not maintaining the hemolytic activity of the first, still had titers which were approximately only double those of the original sera at the end of the eighth week. Sample two at this same time titrated 0.20 cc., and maintained this titer through the seventeenth week. The difference between samples one and two at the end of the first week was 0.06 cc. and the same difference was shown at the end of the thirteenth week. Sample three showed a titer of 0.20 cc. at the end of the eighth week. The preservative action was about equal at the end of the ninth week for the last two samples, when the original titers of the two sera are considered.

Twelve per cent sodium acetate solution: Our results would indicate that at room temperature this method was not useful, as the titer had dropped 0.12 cc. at the end of the first week. At refrigerator temperature, however, it preserved a titer approximately equal to that of the original sample for two weeks, at the end of four weeks there was a difference of only 0.04 cc. and the difference at the end of nine weeks was only 0.07 cc. Such a complement could be considered usable for a period of nine weeks when the original sample was sufficiently active.

Ten per cent sodium acetate with boric acid: The first sample, stored at room temperature, held its original titer for one week. During the next two weeks the titer dropped only 0.02 cc., but by the end of a month it was three times the original titer. The second sample showed, for the first five weeks, an even titer which dropped only 0.04 cc. from that of the same serum when fresh. Stored in the refrigerator, the first sample maintained its original titer for three weeks. At the sixth week, the titer doubled but was still high, and this tripled during the seventh week. An even titer, showing a difference of 0.02 cc. from that of the

same serum when fresh, was maintained for four weeks by the second sample. At the end of the eleventh week the difference was 0.06 cc. The results would seem to indicate that a reasonable titer could be maintained even at room temperature for a period as long as three weeks. Storage at refrigerator temperature gave better results. When both samples are considered, a drop of only 0.06 cc. was noted over a period of six weeks' storage. If the original serum had a high titer, this method should be considered valuable for a storage period of at least six weeks.

Saturated sodium chloride: Complement treated in this manner held its original titer for two weeks at refrigerator temperature. By the sixth week the titer more than doubled, but due to the originally high titer the complement was still relatively active. At room temperature, the original titer was maintained for one week; it more than doubled during the second week, and by the third week reached the selected limit.

Ten per cent sodium chloride with boric acid: A considerable difference between the two samples held at room temperature was noted. The first sample deteriorated rapidly. The second varied only 0.02 cc. from its original titer over a period of five weeks.

Held at refrigerator temperature, the first sample kept its original titer for two weeks, but doubled it during the third week. At the end of the seventh week, a difference of 0.10 cc. was noted. The second sample showed only a difference of 0.02 cc. for the first six weeks and 0.06 cc. at the end of the eleventh week. The two samples had the same titers at the seventh week but the actual loss of potency was greater in the first sample. This further emphasizes the fact that only sera of high titers should be preserved and stored, an observation frequently made during this experiment.

Seventeen per cent sodium chloride: This preserved complement at approximately its original titer both at room and refrigerator temperature for one week, and by the end of the second week the titers were still equal, but 0.06 cc. poorer than those of the original samples. After that, the titer of the sample stored at room temperature was far inferior to that of the sample held at refrigerator temperature, the latter decreasing 0.09 cc. by the end of thirteen weeks and requiring seventeen weeks to reach the selected limit. Whether a difference as great as 0.09 cc. would have been reached in thirteen weeks if the original sample had been less active than 0.04 cc. could not be determined, but a reasonable amount of preserving action seemed assured for at least six weeks when the sample was stored in the refrigerator.

Twenty-five per cent sodium chloride: For the first two weeks, at room temperature, the titration value of the sample remained within 0.04 cc. of the original value. By the third week, the titer differed from the original by 0.09 cc. At refrigerator temperature, the titer remained approximately equal to that of the original sample for two weeks. It was slightly more than doubled at the end of the sixth week. Although doubled, the titer was of such strength that it might be considered usable. Again the original sample was of high titer and the preserving action should be considered with reference to the fresh complement.

Ten per cent sodium chlorate with boric acid: No preservative action on complement was shown at either room or refrigerator temperature.

Four per cent sodium fluoride: Complement activity was not preserved at room temperature, but when stored in the refrigerator, a titer equal to that of the same serum when fresh was maintained for two weeks.

At the end of the sixth week the titer was nearly double that of the original sample, but still usable.

Five per cent strontium chloride with boric acid: No preservative action on complement was shown at either room or refrigerator temperature.

Five per cent magnesium sulfate: The original titer of the complement, at refrigerator temperature, was maintained for only one week; during the second week the titration value doubled, and by the fourth week it was over 0.30 cc. Room temperature storage proved of no value.

Freezing: Complement frozen in the ice compartment of an electric refrigerator was usable at the end of the first week, but by the end of the second week the titer was more than double that of the original sample of serum, a decrease of 0.14 cc.

All of the above results are shown in Table XII.

Dehydrated Complement: Complement, dehydrated for only three hours, and stored at room temperature, had a titer of over 0.30 cc. at the end of the first month, while storage in the refrigerator produced a titer of 0.26 cc. at the end of the first month and a titer of over 0.30 cc. at the end of the second month. When dehydration was prolonged to twenty-two hours* the titer was equal to that of the same serum when fresh at the end of the first month. A second sample gave a similar titer at the end of four months. These samples were held in the refrigerator and the results are listed in Table XIII. Unsalted complement, dehydrated twenty-two hours and restored to its original volume, was preserved with 17 per cent sodium chloride and stored in the refrigerator. The first two weeks a difference in titer of 0.05 cc. was noted between the preserved complement and the original sample.

* Through the kindness of Dr. Harry Eagle of the Johns Hopkins Hospital, four samples of complement (two unsalted, one salted with 0.15 cc. of 17 per cent sodium chloride solution, and one salted with 0.30 cc. of 17 per cent sodium chloride solution) were dehydrated for twenty-two hours.

From the fourth through the ninth week the hemolytic titer was one-half (or a difference of 0.10 cc.) that of the original sample, as may be seen in Table XIV.

Salted dehydrated complement: The sample of complement salted with 0.15 cc. of 17 per cent sodium chloride solution, dehydrated for twenty-two hours, and held at refrigerator temperature, had a titer at the end of the second month equal to that of the same sample of serum when fresh. The sample of complement salted with 0.30 cc. of 17 per cent sodium chloride solution, dehydrated and refrigerated as above, at the end of the fourth month also had a titer equal to that of the same sample of serum when fresh. The results on dehydrated complement are given in Table XIII.

Complement salted with 0.15 cc. of 17 per cent sodium chloride solution followed by dehydration for twenty-two hours and restoration to isotonicity, was preserved in an atmosphere of carbon dioxide at refrigerator temperature. During the first six weeks, the hemolytic activity was one-half that of the original sample, differing by 0.10 cc. Results are given in Table XIV.

Lyo Complement: The sample of Lyo complement used, the original titer and age of which was unknown, gave a titer of 0.12 cc. When a solution of this, restored to its original volume, was preserved by freezing in the ice compartment of an electric refrigerator, the titer was more than double that of the original restored complement at the end of the first week. By the third week a titer of over 0.30 cc. was attained. Lyo complement, restored to its original volume, preserved with 10 per cent sodium chloride with boric acid, and stored at refrigerator temperature, varied from its original titer by 0.08 cc. at the end of the first

week. By the end of the third week, a difference of 0.10 cc. was observed. During the next five weeks its hemolytic power was reduced one-half. These results are tabulated in Table XIV.

# IV

## DISCUSSION

Effect of Physiological Saline: In hemolysin titration, saline prepared from tap water gave a decidedly higher titer than that prepared from distilled water. In complement titration, there was not such a difference in titer. Mason and Sanford (34) stated that the optimum pH for amboceptor titration was 7.0 to 8.4. The highest hemolysin titer in our experiments was obtained with a tap water saline at the highest pH reached, 8.2. Low titers were secured with a Ringer's solution unheated at a pH of 7.9, but with this exception there did seem to be some correlation between pH of the saline and hemolysin titration, a satisfactory titration not being obtained unless the pH was above 7.0. Two tap waters gave higher titers, however, than did the phosphate-buffered saline of Mason and Sanford.

Hemolysin titrations were higher when made with saline that was heated in flowing steam for thirty minutes as compared with unheated salines, and in some cases they were still higher in autoclaved salines. In the complement titrations, where the salines were nearer neutrality, there was little difference in titer between heated and unheated salines. Although a steamed saline as recommended by Kolmer (27) gave good results, a higher hemolysin titer was secured with autoclaved saline made from our tap water.

The addition of magnesium salts to a distilled water saline after Kellogg and Wells (26) gave the highest complement titer secured. In this case the pH of these two salines remained the same, although the titer was doubled. Our tap water saline was also improved by the addition of magnesium chloride. The addition of phosphates to our distilled water saline reduced the amount of complement needed about one-half. A modified Ringer-

Tyrode's solution similar to that recommended by Brooks (7) gave such a precipitate that it could not be used.

In complement titrations where a satisfactory saline was used for diluting all reagents but one, the use of an inferior or superior saline, either for making up the volume or for diluting hemolysin or complement alone, did not result in any uniform difference between distilled and tap water salines or between heated and unheated salines. When the corpuscles alone were diluted with the various salines the titers obtained with steamed salines were higher than with unheated, and the autoclaved salines gave higher titers than the steamed salines, indicating that the increased evaporation and concentration of salts did affect the titer in this instance.

Freshly prepared solutions were used in all of our experiments except in the last hemolysin titration where the saline was used a second day. The results were equally as good, and it is believed that a tap water or buffered saline could be kept for some time without changing materially. In view of the usual recommendations that saline be prepared with sodium chloride and distilled water, it is suggested that attention could well be given to the local waters available and possibly some uniform saline adopted for diluent in any standard proposed for complement fixation reactions.

Surface Tension Measurements: The difference in time drop in the surface tension of sera from individual males placed in the same group according to titer, and the fact that the differences between the most potent and least potent sera were no greater than those found in the same titer group, show that the surface tension measurements were without significance. No definite relationship between titers and surface tension measurements of either pregnant or non-pregnant female sera could be established.

When all three groups are considered, it is impossible to assign any value to surface tension determinations as compared with titers, although the two female groups tended to show greater time drops. The meaning of this observation is obscure. Pregnancy might interfere with the surface tension of sera but that could not account for the non-pregnant group. Time drops of the same value were obtained in the three groups irrespective of titer. If all time drops and titers are compared regardless of their source, such wide variations are apparent that no definite correlation can be found.

Animals Left with and without Feed for Eighteen to Twenty-four Hours Before Bleeding: From the thirty-one samples of pooled sera used for comparison of animals left with and without feed for eighteen to twenty-four hours before bleeding, an average difference of .013 could occur by chance alone in only about one case out of thirty. Hence, it is assumed that the difference resulted from causes other than chance, and is, therefore, significant. This difference favors those animals from which the feed was removed eighteen to twenty-four hours before bleeding. The standard error was $\pm$ .006.

Male and Non-pregnant Female Complement Titers: From the complement titration of thirty-four male and twenty-eight female samples of blood used for a comparison of titers in males and non-pregnant females, the observed difference was not significant when compared to its standard error. The standard error was $\pm$ .0094.

Blood Pooled Before Clotting and Sera Pooled After Clotting and Refrigerating Twenty-four Hours: In the thirty-one samples used for comparison of sera pooled before and after clotting and refrigeration for twenty-four hours, an average dif-

ference of .0077 could occur only by chance in about one case out of thirty; therefore, it is assumed that the difference resulted from causes other than chance and is therefore significant. This difference favors pooling three samples of blood before complete clotting. The standard error was ± .00343.

Production: A true comparison can hardly be made between male and female complement titers from the feeding experiments. The two sexes were placed together in the same cages and as a result between the fourth and sixth months the females became pregnant. This accounts for the almost invariably lower titers of the females. Because of the interference of pregnancy with complement potency the results obtained with females with regard to feeding, age, weight, temperature, sunlight, and litter mate comparisons were not significant. From the experiment it was hoped some information could be obtained as to the influence the above factors might have on complement titer. Rich (47) reported pregnancy to have no effect on the complement content of guinea pig serum, while Browning (8) made the statement that a deficiency in complement is associated with pregnancy. Our results substantiate the latter report.

In general the complement content of male animals increased as they became older. Six of the eleven animals that lived to the completion of the experiment suffered no drop in titer during the last several months. The drop in titer manifested by the other five may be considered as due to individual variations, as its distribution could not be correlated with the cages in which the pigs were raised.

Results of these experiments indicate that guinea pigs should weigh not less than 450 gms. and preferably 600 gms. or more before the most potent titer is reached. Cummer's (10) recommendations that guinea pigs

weigh 400 gms. or more before used as a source of complement compare closely with the information gained from these studies, although the present studies indicate the use of larger animals. Simmons (56) alone definitely states that full-grown healthy males should be used. The author's results are somewhat contrary to those of Friedberger and Gurwitz (14) who indicated that the differences in complement titer of animals weighing between 150 and 600 gms. were independent of age and weight. Kolmer also (29) found little difference in hemolytic activity between a group of twelve animals weighing 200 to 280 gms. and another group of twelve averaging 400 to 580 gms. when they were fed, bled, and the sera treated alike and titrated at the same time. The sex and titer were not given, however, and thus it would not be possible to reconcile his results with ours.

The report of Hyde (25) that there was no appreciable difference between the complement content of young and mature animals was not borne out by the results of these experiments on male animals, which do substantiate the work of Osborn (42) with rats. The fact that new-born animals may have the same titer as the mother, as reported by Friedberger and Gurwitz (15), may be considered to coincide with the present findings, as at the time of birth the mothers almost without exception showed a very low complement content. In a discussion of the complement potency of guinea pigs, Browning (8) stated that there was a marked change during the first three months of life. The results of this work indicate that for most males an age of from five to seven months must be reached before a titer as great as 0.1 cc. is attained, although some animals reached that titer as early as the third or fourth month.

Female litter mate comparisons were difficult to evaluate due to pregnancy. Wide variations in animals of the same litter were noted as well as variations between the litters. When variations as great as those found between individuals of the same litter and between members of different litters occur, significant deductions can hardly be made.

Male and female litter mate comparisons were obscured by pregnancy. Only a limited number of observations could be made on male litter mates. The titers of members of the same litter were in rather close agreement, while differences between the litters were wide. The number of comparisons was too few to reach definite conclusions.

None of the females, regardless of ration fed or where housed, reached and maintained a satisfactory complement strength. Pregnancy was again the controlling factor. Temperature, diet, and sunlight effects on females of the various cages could not be evaluated to show any significant differences.

The recommendations of the United States Department of Agriculture (59) and Austin (2) for feeding guinea pigs were followed in that diets were well balanced. Rich (47) and Browning (8) were both of the opinion that diet had little to do with complement content, and Simola and Brunius (57) reported that neither A or C avitaminosis nor the feeding of vitamin D would affect the potency of guinea pig complement. A deficiency in vitamin A was accompanied by a drop in complement titer of rats according to Osborn (42). The present work cannot be compared with that on vitamins but these results would tend to show the probable lack of effect of feed on complement.

Ruediger (51) using four different diets could observe little difference in their effect. Kolmer (29) maintained that to obtain a good complement it was only necessary to keep the animals in good health and in general the same conclusion was reached by the author after feeding animals

for a year. The results, however, favor the ration fed to cages 3 consisting of a commercial feed supplemented by cabbage every other day.

Guinea pigs are generally raised indoors. A report of the United States Department of Agriculture (59) points out that the temperature should not drop below 65° F. The author's results would not conform entirely to this recommendation as far as complement titer is concerned. The cold weather affected only the young pigs which would not survive long after birth unless kept well covered with bedding. For no apparent reason, the majority of the animals which did not survive the experiment were housed indoors.

Preservation: When room temperature was used to store complement preserved in an atmosphere of carbon dioxide, the samples failed to compare in potency with those that had been stored at refrigerator temperatures. These results obtained by using an atmosphere of carbon dioxide do not agree entirely with those of Valley and McAlpine (61), but carbon dioxide can be considered as preserving complement in a relatively active state for at least three weeks at refrigerator temperature.

A comparison of sodium acetate and sodium chloride as preservatives with storage at refrigerator temperatures, showed no marked superiority of one over the other in one instance (samples having an original titer of 0.10 cc.), while in another instance slightly better results were obtained with sodium acetate. Sonnenschein (58) found complement better preserved with sodium acetate than with sodium chloride. If the fresh complement had a high titer, our results would indicate that if kept in the refrigerator a reasonable hemolytic activity could be maintained for at least six weeks when preserved with sodium acetate or sodium chloride with boric acid. When preserved the same way and held at room temperature, three out of four samples were usable as long as three weeks. Our findings would indicate that

sodium chlorate is not a useful method for the preservation of complement. These results do not substantiate those of Shirvindt and Liberman (55) who advocate the use of 10 per cent sodium chlorate with 4 per cent boric acid, but are more in accord with their results indicating adequacy of 10 per cent sodium acetate with 4 per cent boric acid.

The results obtained support the opinion of Ruffner (52) that 10 per cent sodium acetate, or sodium chloride, with 4 per cent boric acid are among the better methods for preserving complement. However, our findings would not indicate that complement kept at room temperature maintained full activity for three to four months when preserved with 10 per cent sodium chloride and 4 per cent boric acid as reported by Ginsburg and Kalinin (18). Their samples, however, were stored in darkness while ours were kept in a glass cabinet exposed to ordinary light in the room.

Magnesium sulfate had only limited preserving power, and that only at refrigerator temperature. Contrary to the report of Ginsburg and Kalinin (17) strontium chloride failed to show any preserving action, our results agreeing with Ruffner (52). Saturated sodium chloride gave results that are nearly in accord with those of the National Institute of Health (36), and indicate that a reasonably strong complement can be preserved for a six-week period if the original titer is high.

A satisfactory complement was preserved six weeks by treating with sodium fluoride and refrigerating, results materially better than those reported by Ronchese (49).

When complement was frozen in the ice compartment of an electric refrigerator, its titer would indicate that it could not be considered usable more than a week. Our results do not agree with those of Browning and Mackie (9) who used a storage temperature of -12° C.

A 12 per cent sodium acetate solution preserved complement at a usable titer for nine weeks when stored in the refrigerator. This length of preservation is not equal to that found by Rhamy (46) but our results, contrary to those of Ruediger (50), do indicate that the method is valuable.

When a 1:20 dilution is desired our results would indicate that 17 per cent sodium chloride, as reported by Kolmer (32), was a useful method. A reasonable amount of preserving action seemed assured for at least six weeks at refrigerator temperature. Although approximately half of the complement activity was lost by the sixth week when preserved with 25 per cent sodium chloride and stored in the refrigerator, the titer was such that it was usable and the results agree with Kolmer (30).

The activity of complement was preserved little if any when frozen and then dehydrated for only three hours. When unsalted complement was dehydrated twenty-two hours, a titer equal to that of the original sample was maintained for four months at refrigerator temperature, which is the longest time that any dehydrated complement was stored. Our results on dehydrated complement agree with those of Flosdorf and Mudd (13), Eagle, Strauss, and Steiner (12), and Boerner and Lukens (5). This method of preservation was superior to any of the methods discussed above.

Samples preserved by salting when restored at the end of two and four months had a titer equal to that of the fresh serum. This again would indicate that salting and dehydration of complement was superior in preservative action to any of the chemical methods tested above.

The next problem was to determine whether this dehydrated complement, after it had been restored to its original volume, could then be preserved by some of the same means which had been effective with fresh complement.

If so, some of these methods might prove valuable for the small laboratory where there would be some complement remaining after restoration and immediate use. Our results would indicate that when freezing in the ice compartment of an electric refrigerator was used, the potency was not preserved to any great extent and these results are approximately those found when samples of fresh complement were preserved in a like manner.

Ten per cent sodium chloride and boric acid did not preserve restored complement as well as it preserved undehydrated serum. The same results are indicated with carbon dioxide and with 17 per cent sodium chloride which was slightly superior to the other methods. However, the complement might be considered as usable, in extreme cases, after preservation and storage at refrigerator temperature by any of the three methods for at least three weeks, although half the complement activity was lost in all cases. The original titers of the dehydrated complements were also somewhat lower than those of some of the complements preserved with chemicals.

# V

## SUMMARY AND CONCLUSIONS

Physiological salines prepared with unheated tap water unbuffered, or distilled water buffered with phosphates, gave decidedly higher hemolysin titers than when made with distilled water, and gave further increases upon heating. Heated tap water buffered as a modified Ringer's solution also gave high hemolysin titers. This was possibly correlated with increased pH and a satisfactory titer of unbuffered salines required a pH above 7.0.

There was little correlation of complement titers and pH of unbuffered salines, and no uniformly higher titers were found upon heating except when diluting corpuscles alone. The addition of 0.1 gm. magnesium chloride to tap water reduced the complement required somewhat, while with distilled water the addition of phosphates or 0.1 gm. magnesium chloride reduced the complement required to half. The variation in waters indicates the desirability of a uniform saline, and the results suggest the stability of a satisfactory tap or buffered saline.

The surface tension, expressed as time drop, of fifty-two samples of guinea pig complement measured with the du Noüy tensiometer, showed no correlation with hemolytic activity.

The removal of feed for eighteen to twenty-four hours before bleeding the guinea pigs for complement gave a slightly more active complement. Sera obtained from blood pooled before complete clotting gave slightly higher titers than when pooled after clotting and refrigeration for twenty-four hours.

Extreme cold did not interfere with the complement content of animals reared outdoors during a winter of freezing weather, whereas excessive heat and humidity in summer killed mature animals, death being due to suffocation.

The sera of all male animals gradually increased in complement titer after three months, and this titer remained rather constant after six months. Males weighing 600 gms. or more had the highest complement titer and were generally between six and seven months old before reaching a titer of 0.1 cc. The titers of male litter mates agreed fairly well although wide variations occurred between litters. Males fed a commercial prepared feed, with cabbage every other day, reached a good titer earlier than those on other diets. No definite effect was noted of housing indoors or out.

Females invariably showed a lower complement titer than males of the same age after the fourth to sixth month, because of pregnancy. Accordingly pregnant guinea pigs were undesirable as a source of complement and this influence made it impossible to reach conclusions regarding the effect of age, weight, litter mates, feeding, sunlight, or temperature upon females. No significant differences were noted between male and female complement titers when pregnant animals were eliminated.

Unpreserved complements stored at room temperatures were not usable when first titrated after three days, while the titer of refrigerated samples did not drop as rapidly. Refrigerator temperatures were also much superior for the storage of all preserved complements.

Strontium chloride and sodium chlorate with boric acid did not prove of any value as a preservative for complement at either room or refrigerator temperatures. Magnesium sulfate had limited preserving power at refrigerator temperature.

When stored at room temperature, a satisfactory titer of complement was maintained for three weeks by preserving with 10 per cent sodium acetate with boric acid. One of two samples of 10 per cent sodium chloride with boric acid preserved for three weeks, 17 and 25 per cent sodium chlo-

ride for two weeks, and a saturated sodium chloride solution preserved satisfactorily for one week.

Complement stored in the refrigerator was active enough for use for not more than nine weeks when preserved with a 12 per cent solution of sodium acetate and for six weeks when preserved with 10 per cent sodium acetate (or sodium chloride) with boric acid, a saturated sodium chloride solution, 17 and 25 per cent sodium chloride, or 4 per cent sodium fluoride.

A rather high hemolytic titer was maintained for at least three weeks when complement was preserved in an atmosphere in which the oxygen had been almost completely replaced by carbon dioxide, and stored in the refrigerator.

Freezing maintained complement at a usable titer for only one week and failed entirely to preserve restored frozen-dehydrated complement.

Preservation by freezing and dehydration of salted and unsalted complement for twenty-two hours, followed by storage in the refrigerator, gave the best results, maintaining the original titer for four months, the duration of storage.

Seventeen per cent sodium chloride was the most desirable method of those tried for preserving left-over restored frozen-dehydrated complement, if a dilution of 1:20 was desired. Carbon dioxide was found satisfactory when a 1:10 dilution was wanted.

Most methods for the preservation of complement did not maintain the original titers upon storage. Accordingly, the fresh complement should have a high titer and this was best assured by using full-grown, healthy, well-nourished male or non-breeding female guinea pigs.

# VI

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