SEROLOGICAL STUDIES OF THE REPTILIA

by

Glenn C. Bond
A. B. Phillips University, 1927
A. M. University of Kansas, 1933

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Advisers:

Noble P. Sherwood,

Henry T. Macy

Edward H. Taylor

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INTRODUCTION

This introduction serves as a discussion of the literature, theories, ideas, and suggestions in regard to the subjects related to the experimental portion of this work.

No attempt is made to cover all of the related work, but enough will be considered to give the current and some of the original theories of these principal subjects.

The experimental work is divided into five sections, each dealing with a separate phase of the work done, yet all are correlated to the end of attempting to show the existence of some similarity of the bloods of the Reptiles and the Mammals, although their Biological relationship is quite remote.

I. HUMAN BLOOD GROUPS

A. History

The discovery of human blood groups by Land-steiner (1) in 1901 was an outgrowth of a series of immunological studies which had been going on for some time. The phenomena of lysis and agglutination of bacteria and red cells had been described and even extended to the distinguishing of iso and hetero-hemolysins and agglutinins. The identification of iso agglutination probably gave impetus to the work which ultimately gave us blood grouping.

Added interest to this field of investigation was brought about by many favorable and as many more unfavorable reported results of transfusions. This aspect of therapeutics had undergone a series of favorable and unfavorable stages in its development and, as a whole, the results obtained had been very unsatisfactory. The idea of the transfusion of blood from a healthy to a sick individual seemed very practi-cal, but results were far from pleasing. So great was the disfavor to this practice that a period of approximately fifteen years elapsed after the dis-covery of human blood groups before it found its rightful place in the practice of medicine. This placement may be attributed directly to the World War, when a great many cases arose where transfusions were

of real value.

Landsteiner was able to classify the small series of bloods examined by him into three groups on the basis of isohemagglutination. These he termed groups A, B, and C. Group A serum agglutinated cells of group B but not group C, while A cells were agglutinated by serum of both B and C groups. Group B serum agglutinated the cells of group A but not of group C, and group B cells were agglutinated by serum of both groups A and C. Group C serum agglutinated cells of groups A and B, but group C cells were not agglutinated by the serum of either group A or B.

The following year von Decastello and Sturli (2) described several sera in which the serum did not agglutinate the cells of any group but the cells of this group were agglutinated by the serum of all other groups. Additional investigation showed these two belong to an additional group. Confirmation of these four groups was made by several investigators and in 1907 Jansky (3) made the first classification of these four groups and called them I, II, III, and IV. In 1910 Moss (4), not having seen the classification of Jansky, formulated a second classification of these four groups and in his classification reversed groups I and IV of Jansky.

More recently a grouping has been devised on the
basis of the agglutinogen content of the cells of the
four groups. These four groups are called O, A, B,
and AB. O indicates the absence of agglutinogens in
the cells of this group. The letters A and B are used
to designate two agglutinogens. The type name indi-
cates which of these agglutinogens are present in the
cells of the respective groups. This grouping has been
recommended by a special health committee of the League
of Nations and the American Society of Immunologists.

At the present time then we have in use three
methods of classification, the relationship of which
can best be expressed in chart form.

Jansky	I	II	III	IV
Moss	IV	II	III	I
New	O	A	B	AB

This new classification is used quite extensively
in the current literature and will probably replace the
other two. The use of the Jansky and Moss classification
has caused some confusion and occasionally disastrous
results in transfusions due to the reversal of groups
I and IV. The new terminology will be used exclusively
throughout this work.

Landsteiner in his early work postulated the

existence of the two agglutinable substances A and
B, and the existence of two agglutinating substances
now termed alpha and beta. He further postulated
that agglutination between blood types was due to
an A-alpha complex or to a B-beta complex, and that
these factors were present in the blood in a re-
criprocal arrangement i.e. A and alpha or B and beta
could not exist in the same blood. The following
table illustrates the distribution of these agglutin-
able and agglutinating factors.

Cell Agglutinogens   O   A   B   AB
Serum Agglutinins $\alpha\beta$ β α O

With an increase in the number of reported in-
vestigations of the determination of blood types,
irregularities from the four types were noted. It
was first observed by von Dungern and Hirschfeld (5)
that groups A and AB could be divided into two sub-
groups on account of the differences in the A factor.
These factors are called A1 and A2 with their corre-
sponding agglutinins α_1 and α_2. The proof of the ex-
istence of these subgroups has been established by
absorption experiments. Detection of these subgroups
is also possible by using sera prepared against human

cells. Another irregular factor which has been de-
scribed by several investigators as being present in
some human blood, with nearly equal frequency in all
four groups, is the factor P.

In 1928 Landsteiner and Levine (6) by means of
prepared immune sera, described several new agglutin-
able factors in human blood. The most significant
of these were called the M and N factors. The factors
are demonstratable only by the use of specific immune
agglutinating serum, prepared usually in rabbits. The
antiserum for M can be produced by injecting rabbits
with blood of group O and type M+N- ; and the antiserum
for N by injecting group O blood of type N+M-. Normal
antibodies specific for M and N are not present in
normal human serum. Upon examining numerous bloods
of all of the four types with the two immune sera
described above, and , having been previously ab-
sorbed with a suitable blood lacking the factor in
question, three types are found. These are M+N+,
M+N-, M-N+. Although thousands of bloods have been
examined, a blood of the type M-N- has never been
observed.

Since these three types have been found to be
transmitted in a genetic manner as are the groups
O, A, B, and AB, this offers possible combinations

to secure twelve sorts of human blood. It is possible
that additional factors may eventually be included
and a greater number of different bloods described.

The present status of the grouping of human bloods
is as follows. Four groups O, A, B, and AB, with the
possibility of two subgroups for types A and AB, and
the possibility of three types for each group, namely,
M+N+, M+N-, and M-N+. A few other factors have been
described but at the present are of little importance
as compared to those mentioned.

B. Heredity of Blood Groups

In 1908 Epstein and Gottenberg (7) suggested that
the incidence of the four well defined blood groups
might be subject to the laws of Mendelian inheritance.
In 1910 von Dungern and Hirschfeld (8) established,
by a study of blood groups in families, that the
isoagglutingens A and B are inherited as two pairs of
Mendelian factors, the isoagglutinogens A and B being
dominant to their respective isoagglutinins α and β .
On this basis a specific agglutinogen could not appear
in a child unless it was present in the blood of one
or both of its parents.

This theory was accepted and apparently confirm-
ed by many workers, but as a greater mass of sta-
tistical evidence was accumulated, it became evident,

that this two independent factor theory of von Dungern and Hirschfeld would not fit the statistical results which had been obtained.

In 1925 Bernstein (9) advanced his triple allelomorph theory of the heredity of blood groups, which seems to agree with the statistical results obtained by scores of workers.

This theory assumes the existence of three allelomorphic genes, A, B, and O, one of which is present in each member of a certain pair of chromosomes. Genes A and B are dominant over O. Since each somatic cell possesses two of these genes, one derived from either parent, there are six possible genetic types or genotypes. From these six genotypes it is possible to derive four phenotypes or the four demonstratable blood groups. The following table shows the six possible genotypes and their relation to the blood groups or the four phenotypes.

Genotypes   Phenotype
(one gene from each parent)   (Blood Group)

OO--O

OA   A
AA

OB   B
BB

AB --- AB

The inheritance of these factors can best be illustrated by an example. Let us see what offspring are possible from the mating of the two genotype parents OA and OB.

```
    O   A   O   B
     \ / \ / \ /
      X   X   X
     / \ / \ / \
    OO  OB  OA  AB
```
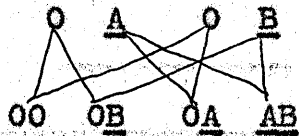
Remembering that A and B are dominant over O, it is evident that there is a possibility of all four phenotypes resulting from this mating. In like manner the possible resulting phenotypes from any genotypic cross can be calculated. The following table shows the possible and impossible phenotypes from any possible genotypic match.

Parents	Possible types	Impossible types
O + O	O	A,B,AB.
O + A	O,A.	B,AB.
O + B	O,B.	A,AB.
A + A	O,A.	B,AB.
A + B	O,A,B,AB.	
B + B	O,B	A,AB
O + AB	A,B.	O,AB.
A + AB	A,B,AB.	O.
B + AB	A,B,AB	O.
AB + AB	A,B,AB	O.

Two rules of inheritance of blood types can be formulated from the above calculated results. 1. The dominant agglutinogens A and B cannot appear in the offspring unless they are present in at least one parent. 2. An O parent cannot have an AB child and an AB parent cannot have an O child. This first rule of dominance was previously stated in connection with the theory of von Dungern and Hirschfeld and is common to both theories. The second rule is called the Bernstein rule and is peculiar to this theory only. Statistical evidence bears out both of these rules.

The M and N factors previously mentioned have been a subject of investigation in regard to their hereditary properties. Investigations have proven that the agglutinogens M and N are genetically determined by two allelomorphic genes which can produce only three genotypes, MM, MN, and NN. The factor M or N does not appear in the blood of a child unless present in at least one parent and a parent of type M cannot have a child of type N, nor can a parent of type N have a child of type M.

All of these rules concerning the O, A, B, M, and N factors have been sufficiently checked by statistical

data and at the present time are quite universally accepted. Two other theories have been advanced but do not explain statistical results as well as the Bernstein theory, therefore do not hold a place in the study of the heredity of human blood groups.

C. Blood Groups in Forensic Medicine

The available information concerning blood grouping has been utilized in the field of forensic medicine along two principal lines. 1. The determination of non-paternity in alleged cases of illegitimacy. 2. In criminal procedures for the identification of the blood type of spots of blood and other stains of human origin. These two phases will be discussed in the order given.

Shortly after the discovery of blood groups by Landsteiner in 1901, additional serological methods were introduced into the field of forensic medicine. By 1910 von Dungern and Hirschfeld (8) had advanced their theory of heredity of human blood groups and evidence was being accumulated in regard to the one basic idea this theory advanced. This fact was that the isoagglutinable factors A and B are never present in children unless they are found in one or both of the parents.

Although the newer theory of Bernstein has replac-

ed the now disproven theory of von Dungern and Hirsch-
feld the essential fact as mentioned above has not
been altered in any way, so that the accumulated
evidence is still useful.

The possibility of using this information is
best expressed in the previously presented table
which shows what types of children are possible and
which types are not possible from various matings of
parents.

It can be seen from this table that exclusion of
paternity can be secured in only a percentage of the
cases. Boyd and Hooker (10) have calculated the
percentage of exclusion for each type and found them
to be as follows. Type O- 1 in 5, type A- 1 in 17,
type B- 1 in 7, and type AB- 1 in 2. The average
chance of excluding paternity is about sixteen
percent. Wiener (11) estimates the exclusion per-
centage in Germany to be somewhat higher, it being
about eighteen percent.

Schiff (12) in 1929 compiled statistics obtained
in Europe, where determination of blood types had been
accepted as legal evidence, with the following results.

	Number of cases	Paternity exclusions	Percent of exclusions
Germany	4,519	353	7.8
Austria	700	63	9.0
Danzig	600	39	6.5
Denmark	50	6	12.0
Denmark	500	64	12.8
Sweden	259	17	6.8
Norway,Switzerland, Lithuania	37	4	10.8
Total	6,665	546	8.2

This table shows the comparatively wide use of
blood grouping tests in Europe and that a rather high
percentage of exclusions has been obtained.

The discovery of the additional agglutinogens M
and N by Landsteiner and Levine in 1928 (13) has in-
creased the possibility of exclusion considerably.
These additional agglutinogens may be utilized to
about the same extent as the A and B factors. Although
the technic is more difficult, statistics have born
out the calculated results from various matings as
expressed in the following table.

Parents	Children possible	Children not possible
M + M	M.	N,MN.
N + N	N.	M,MN.
M + N	MN.	M,N.
M + MN	M,MN.	N.
N + MN	N,MN	M.
MN + MN	M,N,MN.	

The utilization of all four possible factors, A, B, M, and N, has increased the possibility of paternal exclusion to approximately thirty-three percent. Landsteiner (14) states that the chances for detecting interchange of new-born infants in hospitals is raised to about seventy percent by the additional information, whereas with the A and B factors alone the possibility was approximately fifty percent.

Since it is usually a question of paternity, the following table illustrates the use of blood typing in medico-legal procedures. These tables are after Snyder (15) 1936.

Blood group of child	Blood group of mother	Blood group to which father cannot belong
O	O	AB
O	A	AB

(continued)

Blood group of child	Blood group of mother	Blood group to which father cannot belong
O	B	AB
A	O	O,B.
A	B	O,B.
B	O	O,A.
B	A	O,A.
AB	A	O,A.
AB	B	O,B.
AB	AB	O.

Type of child	Type of mother	Type to which father cannot belong
M	M	N
M	MN	N
N	N	M
N	MN	M
MN	M	M
MN	N	N

The mass of statistical evidence which has been obtained by many workers in support of the above cal-culated results has agreed quite uniformely. In view of this mass of evidence the number of courts accept-ing this information as legal evidence has increased materially in the last few years. The greatest advance

has been made in Europe, and now a few of the states

of the United States are formulating and adopting

laws to introduce this information as legal evidence

in the courts.

The cases reviewed by Schiff (12) have dealt with

the use of the A and B factors exclusively. On the

basis of the evidence of the M and N factors, many

European countries have recently adopted the use of

these factors in affiliation cases. To date these

factors have been utilized in hundreds of cases.

It is rather unfortunate that the courts in the

United States hesitate to recognize the value of this

information in cases of disputed paternity. However

a rather active campaign has been started recently to

bring about the recognition of this scientific aid in

legal procedures. The Journal of the American Medical

Association has aided in this by establishing a section

in the official journal on forensic medicine. This will

keep the question before the members of the medical

profession, so that ultimately they will become more

interested in the possibilities of this phase of

immunology. Professor Wigmore of Northwestern Univ-

ersity School of Law, who is an authority on legal

evidence, states; "At the hands of expert specialists-

but of specialists only- the tests ought to be more

widely known and used in this country, within the

limited scope of their probative value." With these
facts being continually brought before the legal and
medical professions, it is hoped that the American
courts will soon follow the leadership of the courts
in Europe.

The second phase of legal utilization of blood
typing, as has been stated, is in the identification
of stains of human origin.

Landsteiner and Richter (16) in 1903, found that
the agglutinogens and agglutinins in human blood are
quite resistant to aging and drying. From this evi-
dence, and more acquired since this work, the de-
termination of blood type has become a routine
procedure in the full identification of blood stains
of human origin. Many criminal cases have been
settled by evidence obtained in this way.

The precipitin test has been recognized for some
time as a proper procedure for the species identi-
fication of unknown stains, but in many cases this
does not entirely answer the question causing the
dispute. This additional information is of inestim-
able value in increasing the amount of evidence.

Wiener (17) serving as a member of the National
Research Council Committee on Human Inheritance, has
Prepared a series of model laws for states to follow

in their consideration of the adoption of these procedures to secure evidence in legal procedures. These are quoted as follows.

1. Blood Tests in Illegitimacy Actions

"The court, on motion of the defendant, shall order the making of one or more blood grouping tests by a duly qualified physician or other qualified person, and the results thereof shall be received in evidence. The cost of the tests is to be met by the defendant, who may select the expert from a list drawn up by the State Medical Society of individuals who have proved their ability to do such tests. Whenever the court orders such blood tests to be taken and one of the parties shall refuse to submit to such tests, such fact shall be disclosed upon trial unless good cause is shown to the contrary."

2. Blood Tests in Civil Actions.

"Whenever it shall be revelant to the prosecution or defense in a civil action to determine the parentage or identity of any child, person or corpse, the court, by order, shall direct any party to the action and any person involved in the controversy to submit to one or more blood tests, to be made by only a qualified physician or other qualified person, and under such restrictions and directions as the court or judge shall deem proper. Whenever such test is order-

ed and made, the results thereof shall be receivable in evidence. The court shall determine how and by whom the costs of such examinations shall be paid."

3. Code of Criminal Procedure: Identifying Criminals: taking of fingerprints, photographs, and blood grouping tests.

"In order that the courts and public officials dealing with criminals may have accurate information as to the identity of persons charged with crime, there is hereby conferred and imposed upon the chief of police or peace officer performing such function, in each city, town and village, and upon the sheriffs, members of the state constabulary, the village police, the aqueduct police, the state park police and all other peace officers making arrests, the power and duty of causing to be taken upon arrest finger and thumb prints, and if necessary photograph and blood grouping tests, of every person arrested and charged with a felony or with any of the misdemeanors and offenses specified in section ___ of this code."

The States of New York, Wisconsin, Connecticut, Pennsylvania, Maryland, California, and New Jersey have adopted laws similar to the above models and are leading the way for more complete adoption of such laws in the United States.

D. Blood Groups in Medicine

   1. In Relationship to Transfusion

As has been previously mentioned, the practice of blood transfusion has had a very eventful history. Transfusion is an ancient practice which has come down through the years with very little to be said in its favor.

The use of animal blood for transfusion into man was first tried with a variety of results. Some found their patients would recover, others no recovery could be brought about, and in addition many of the physicians lost their donors after a few transfusions. In spite of these results with animal blood, transfusions from man to man were carried out. Many of these ended very disastrously for the recipients and in other cases beneficial results were secured.

In 1668 the practice of transfusion received another setback when such practice was declared illegal in Paris and Rome. There was a revival in the year 1818 due to the work of Blindell in London. This increased interest was manifest for some time, and its influence existed to the time of the discovery of blood groups by Landsteiner.

The description of the four distinct blood types

put the transfusion procedure on a practical basis,
although it was not appreciated until several years
later. Landsteiner suggested that by transfusing
compatible blood, the effects previously found
would be eliminated.

During the World War the practice of blood
typing before transfusions came into its own. The
need for transfusions was great in so many of the
wounded due to such a great loss of blood. After
several fatal results, without paying any attention
to determining the blood types of the donor and the
recipient, routine determination of types was resorted
to with excellent results.

The original methods used for the determination
of compatibility, are still used routinely before
transfusions. The introduction of the other agglutinable
factors are of little importance in this regard. The
M and N factors may be neglected entirely since agglu-
tinins do not exist in human blood for these factors.

Briefly the procedure for the determination of
compatibility is to first determine the type of the
recipient and the type of the prospective donor. The
usual method is to use known type A and known type B
sera and mix a drop of each of these with a drop of a
saline suspension of the donors and recipients cells

respectively. The following table illustrates how the type of the blood under consideration may be determined from this procedure.

	O Cells	A Cells	B Cells	AB Cells
Type A Serum	-	-	+	+
Type B Serum	-	+	-	+

Other procedures which may be used to determine blood types of patients are as follows:

1. Type A cells + Patients serum.

    Type B cells + Patients serum.

2. Type A serum + Patients cells.

    Type A cells + Patients serum.

3. Type B serum + Patients cells.

    Type B cells + Patients serum.

These possible combinations show that blood typing can be done if a person has available either Type A or Type B blood or the serum of both types.

In addition to determining the types of the recipient and the donor, a further check is desirable. This is termed a compatibility test and is performed as follows:

Donors serum + Patients cells

Donors cells + Patients serum

These two setups should be incubated for at least twenty minutes and examined microscopically for traces of agglutination. Some acceptable test for syphilis should be done routinely on the blood of the donor.

The entire test is devised to rule out any iso-hemagglutination or isohemolysis between the two bloods in question. Where this is carefully done, very little danger results from transfusion. Transfusion now is an everyday occurrence and a procedure of very practical importance in medicine.

2. Blood Groups in Relation to Disease

Scores of papers have been written on the question of the relationship of blood groups to disease. Little value has been received from this work up to date, but this may eventually prove to be very useful in regard to incidence, distribution, and the hereditary trans-mission of disease.

Some of the most numerous researches have been done to determine the relation of blood groups to the following diseases: Tuberculosis; Poliomyelitis; Dysentery; Scarlet Fever; Cancer; Syphilis; Leprosy; Pneumonia; Hyperthyroidism; Paralysis; and Allergic conditions. Scores of papers have been written con-cerning the relationship of blood groups to various mental conditions.

Other correlations have been attempted with
the following: Suicide; Sedimentation rate of ery-
throcytes; Age and longevity; Pigmentation; Shape
of cranium; and Papillary pattern of finger tips.

Although as a whole these results are not very en-
lightening as yet, this mass of evidence being accumu-
lated may eventually prove to be of some value.

E. Blood Groups in Lower Animals

The principal part of the following experimental
work has to do with the question of blood groups in
lower animals, and the possibility of a relationship
of these agglutinating and agglutinable factors to the
isoagglutinogens and isoagglutinins of the human species.

The literature concerning this phase of blood
grouping may be discussed according to the following
arrangement.

1. Determination of the presence or absence
   of isoagglutination in lower animals.

2. The possibility of blood groups based on
   the presence of isoagglutinogens and
   isoagglutinins.

3. The presence or absence of agglutinins in
   animal serum for human erythrocytes.

4. The presence or absence of agglutinins in
   human serum for animal erythrocytes.

This literature will be discussed in detail in the

proper section of the experimental work.

The differentation of human and animal blood in the past has been done largely on the basis of the precipitin reaction. The greatest contribution to the study of animal relationships by this method was made by Nuttall (18) in 1904. The value of this procedure has its limitations in the amount of information supplied, yet is extremely useful.

The possible presence of blood groups in animals is of interest from several standpoints. 1. Additional information as to the methods of inheritance of blood groups could be secured. 2. The taxonomic relationship of animals would be more definitely understood. 3. The relationship of lower animals to man would be more definitely proven.

Landsteiner and Miller (19) 1925 offer a valid reason for the increased interest in blood groups of lower animals. "The problem of man's kinship to his closest relatives in the animal kingdom has been studied by the methods of comparative anatomy and paleontology and the results of these investigations are developing into a special branch of learning. When serology provided a new technic for recognizing the biochemical properties which characterize species it became inevitable that the technic should be applied to the

problem of man's ancestry."

More recently impetus has been given to the determination of blood groups of lower animals and their relationship to the blood groups of man through the work of Herman (20) and others in the U.S.S.R. These workers were especially interested in transfusion as a therapeutic measure in the treatment of fine horses. Their success has opened up a new field of veterinary medicine which seems to be quite important.

A second phase of the above work which may prove to be of great importance, is the determination of the presence or absence of agglutinins, in prepared immune serum, for human erythrocytes. It is very possible that some of the disagreeable results obtained following the therapeutic administration of immune serum, may be due to the presence of such agglutinating factors in the animal serum used.

F. Blood Groups in the Science of Anthropology

One of the most important results of the discovery of blood groups within the human species has been its utilization in the study of the racial distribution of blood groups.

The initial work of Hirszfeld and Hirszfeld (21) during the world was in investigating the distribution of blood groups in five hundred soldiers on the

Macedonian front stimulated an inestimatible amount of work by immunologists, geneticists, and anthropologists.

This initial work divided the nationalities into three types.

1. The European type which is made up of the English, French, Italians, Germans, Austrians, Serbians, Greeks and Bulgarians. In this group the A factor is present in from 41% to 48% and the B factor present in from 10% to 20%. On the basis of the biochemical index " $\frac{A + AB}{B + AB}$ " this type has an index of 2.5.

2. In the Intermediate type including the Arabs, Turks, Russians and Jews, the A factor ranges from 37% to 44%, the B factor from 24% to 28% with an index of 1.3.

3. In the Asio-African type including the Madagascans, Senegalese, Annamese and the Natives of India the A factor is present in from 27% to 30% and the B from 28% to 49% with an index of 1.0.

Additional statistical data showed that this grouping was not satisfactory, since some racial types could not be satisfactorily placed in one of these three groups.

Snyder (22) 1929 divides the races into seven types, only the names of which will be given here.

1. European type 2. Intermediate 3. Human 4. Indo-manchurian 5. Africo-Malaysian 6. Pacific-American 7. Australian. He states that this classification will serve only as a working basis and is subject to constant revision.

Snyder has formulated certain laws which he offers as a basis for serological race classification. I repeat those laws here because of a lack of any more recently formulated ones.

1. " Any people being studied from the standpoint of blood groups may be expected to show blood group frequencies similar to those of other peoples known to be related to them."

2. " If any people show blood group frequencies different from those to be expected based on the frequencies of other people known to be related to it, the conclusion may be drawn that the former has undergone racial crossing of some sort which the latter have not undergone."

3. " If any people show blood group frequencies similar to a group of peoples not known to be related to it, the conclusion may be drawn that the former traces back to the latter somewhere in its ancestry, or else that the former has undergone crossing with the latter group or some similar people."

4. " If any people lack one or both of the blood group
mutations, it may be assumed that that people became
isolated from the rest of mankind before the respect-
ive mutations took place, or before they spread very
far."

The application of the study of the human blood
groups to the field of anthropology has been steadily
increasing, so that it has been possible to prepare
blood group maps of the world, which show the pre-
dominance of one group or the other in certain parts
of the world. Available data up to 1935 has been
utilized by Wegman and Boyd (23).

Since the factors A and B, whose presence or
absence determines the four blood groups, are in-
herited as Mendelian dominants, and since their
transmission is not affected by environmental factors,
their racial percentages are of more significance than
any other one single fact in the study of anthro-
pology.

In addition to the study of the distribution of
blood groups in the present races, recent pub-
lications indicate that another field of study is
available to anthropologists, which will increase
our information considerably in regards to the origin
of races.

Matson (24) 1934, and Boyd and Boyd (25) 1934,

introduced a technic whereby it was possible to de-
termine the blood types of mummies. Matson confined
his initial work to Indian Mummies from Arizona, and
later used Egyptian Mummies. Boyd and Boyd have made
an extensive study of Egyptian Mummies. All of this
work is important and very significant and should lead
to quite an extensive survey of available materials.

Candela (26) 1936, taking advantage of the fact
that isoagglutinogens have been demonstrated in most
of the body tissue, used skeletons of thirty Egyptians,
some dating back 3,500 years, and demonstrated the
incidence of blood groups from these. His work co-
rrelates with the results secured from the Egyptian
Mummies by Boyd and Boyd, in that he found a high
percentage of type B.

All of this work has been of paramount importance
to the science of Anthropology and with these initial
stimuli, much more should, and will follow.

II. COMPLEMENT

In 1876 Landois discovered that some fresh animal serum would cause hemolysis of the erythrocytes of other animals, but it was not until later that any attempt was made to explain this action.

Nuttall (27) 1888, Buchner (28) 1890, Pfeiffer and others about the same time observed the bacteriolytic effect of fresh serum on certain organisms.

Many attempts have been made to explain this action, and although much has been learned, no complete explanation is as yet available.

It was found by Buchner (28) that this property was removed from normal fresh serum if it was inactivated at $56^{\circ}C.$ for ten to fifteen minutes. Bordet in 1899 found that this property was due to the presence of two distinct substances, one of which was thermostabile and acted as a sensitizer; the other a thermolabile substance which caused the lytic action.

The names which have been applied to these substances are varied. Buchner called it alexin, Ehrlich and Morgenroth called the thermostabile portion amboceptor and the thermolabile portion complement. The term complement is used principally now in the same manner as used by Ehrlich and Morgenroth. Complement is usually defined as the thermolabile enzyme-like substance found in the blood of animals, which will pro-

duce lysis of sensitized cells. The definition many times includes the statement that it is found in the blood of warm-blooded animals only. Recent researches indicate that it is present in Frogs (29), Eels (30), and this work shows the presence of complement in Snake serum.

A. Origin

Although it is easy to demonstrate the presence of complement in fresh serum, the question as to the origin of this substance in the animal body is one which as yet has not been answered satisfactorily.

Buchner, Hankins and others have suggested that complement is derived from the leukocytes. Evidence which has been accumulated is both in favor of and against this theory. Metchnikoff (31) maintained that the leukocytes and other body cells are a source of complement only upon deterioration of the cells. This conclusion was reached following several experiments in which complement could not be demonstrated in the plasma.

Recently Hyde (29) has demonstrated the presence of complement in the circulating blood by injecting washed sheep cells into a frog, and examined the circulating cells in the vessels of the web of the foot. These sheep cells could be identified for at least two hours. Upon injecting antisheep hemolysin these cells disappeared almost immediately. He further states

that frog serum taken from the clot complements to the
same potency as guinea pig serum.

Another source of complement which has been suggested
is the liver. Again evidence has been presented pro
and con. Ehrlich (32), Dick (33), Nolf and others
have presented evidence in favor, but Sherwood, Smith,
and West (34) concluded that the liver is neither the
only, nor the principal source of complement. The
question as to the origin of complement is as yet un-
decided.

B. Structure of Complement

Complement is undoubtedly protein in nature since
it is antigenic. If serum, which contains complement,
is divided into sero-globulins and albumins, it will
be found that each portion contains a fraction of the
complement. The globulin fraction, according to Ehrlich,
will unite to amboceptor which is fixed to cells and
is called the mid-piece of the complement. The albumin
fraction contains the so called end-piece, which will
unite with the mid-piece attached to the sensitized
antigen and cause lysis. Without the globulin mid-piece
the albumin end-piece cannot unite with the amboceptor,
and in the absence of the end-piece the mid-piece cannot
cause hemolysis. Two or more additional components of
complement have recently been demonstrated.

Culbertson (35) concluded that all of the known

components of alexin can be removed from human serum by filtration through a series of Berkefeld candles. The thermolabile components, one and two, are the first to be removed and are not demonstratable in the serum after 3-6 filtrations. The heat stabile components, three and four, can still be detected after nine filtrations, but all of the components are absent from the twelfth filtrate.

C. Properties of Complement

Complement has been likened to enzymes by Buchner, Ehrlich, and others. Sherwood (36) summarizes the enzyme-like properties of complement as follows.

1. It is colloidal in nature.

2. The presence of a small amount brings about extensive chemical changes in the substrate.

3. It is thermolabile.

4. The reaction is about as reversible as enzyme reaction.

5. The optimum temperature for its action is 37 C.

6. Antienzymes as well as anti-complements have been reported.

7. There is considerable evidence that its activity is interfered with by the end products of its action.

As has been stated, Buchner in 1900 demonstrated the thermolability of complement, when he found that active complement would be inactivated by heating to 56°C. for ten to fifteen minutes. At lower temperatures it requires a greater period of time for inactivation.

D. Distribution

Complement seems to be a constituent of the blood of all normal animals. The amount present varies from animal to animal, and from time to time for the same animal.

In connection with this discussion some observations of Moore (70), Coca (71), Ecker (72), Hyde(37) and others should be mentioned. These investigators studied the blood of a strain of guinea pigs that are different from other guinea pigs, in that complement is missing from their blood. This stock has arisen supposedly as a mutation. By cross breeding it has found that this characteristic is inherited as a simple recessive Mendelian unit. The missing factor seems to be the third component, a heat resistent component.

It is sufficient to say here that a definition of complement which states that it is a constituent of

the blood of warm-blooded animals is not broad enough.
The work of Hyde (29) showing the presence of complement
in Frogs, and the work of Mitano (30) showing the
presence of complement in Eels, and this present work
shows a greater distribution than just in the warm-
blooded animals.

E. Preservation of Complement

At the present the most practical method of
preservation of complement is to keep the serum frozen.
In this way the potency may be retained for some time.
Several other methods have been suggested for preserving
the potency. Dessication has proven to be a valuable
means of preserving the complement titer of guinea pig
serum. Salting methods have been used with variable
results. Valley and McAlpine (38) used various gases
in attempts to preserve complement and found that
carbon dioxide alone exerted a very pronounced pre-
servative action of serum complement. When enclosed
in a carbon dioxide atmosphere in an air tight con-
tainer the complement may be preserved for three to
five weeks without any loss of potency. In another
paper Valley (39) states that this procedure is based
on the hypothesis that complement may be preserved
under conditions which favor reduction and prevent
oxidation.

F. Synthetic Complement

An artificial complement which would act equiva-
lent to normal animal complement would be highly desir-
able. Such a complement was developed by Liebermann
(40), which consisted of a combination of sodium oleate,
proteins (especially globulins), and calcium salts,
forming an emulsion in a colloidal condition. This
artificial complement is inactivated by heat and can be
used as complement in the Wasserman reaction, however
it has not been utilized to any great extent.

III. Heterohemolysins

In the history of the development of the art of transfusion, we find that much of the difficulty encountered was noted to be due to the fact that the mixing of two bloods, many times caused a laking of the cells of one of the bloods used. In 1875 Landois found that the deleterious reactions were decreased if bloods from the same species of animals were used. Subsequent work by Bordet, Ehrlich, Landsteiner and others showed that the laking of cells by a heterologous blood was due to the presence of a natural occuring hemolysin (sensitizer) and the complement of the fresh serum.

The actual mechanism involved aroused much controversy and led to the advancing of at least three theories. Ehrlich and Morgenroth (41) formulated the theory that complement will unite only with the antibody or hemolysin and not with the red cell, but that it acts upon the cells when united indirectly by means of the antibody. Bordet believed that the antibody is not an amboceptor for uniting the cell and complement, but that it sensitizes the cell and renders it susceptible to the lytic action of the complement. Metchnikoff maintained that both the hemolysin and complement are ferments. The amboceptor is likened to

enterokinase and complement to cytase.

Hemolysins or cell sensitizers may be divided into two kinds; normal and immune. The question of immune hemolysins will be dismissed with the statement that these are antibodies which are produced by the injection of foreign red cells into the body of a suitable laboratory animal. The question of normal hemolysins is of more importance here since it has been shown that heterohemolysins for sheep and human cells do exist in snake serum.

The term natural heterohemolysin will be used to designate a natural occuring hemolysin, active on the red cells of an animal of another species.

The presence of natural heterohemolysins in fresh animal serum can be demonstrated by simply mixing the serum with the appropriate cells and utilizing the complement of the fresh serum. If it is desirable to prove further the presence of this factor, it can be separated from the complement of the fresh serum by absorption with appropriate cells at $0^{o}C$. Another method which can be used to secure the heterohemolysin is to heat the fresh serum in order to inactivate the complement. The heterohemolysin being thermostabile will be left in the serum.

The literature in regard to the determination
of the presence of heterohemolysins in various
animal sera will be reviewed in sections II and III
of the experimental work. It is of interest to
mention here that most of the investigations have
revealed, contrary to expectations, that the
occurrence of heterohemagglutinins and heterohemolysins
is largely independent of the degree of zoological
relationship between the species.

The question concerning the reason for the presence
of antibodies in normal serum is one which has not
been satisfactorily answered. Landsteiner (42) suggests
that several possibilities must be considered. The
presence of antibodies such as diphtheria antitoxin is
generally attributed to an unrecognized contact or
infection. The regular occurrence of isohemagglutin-
ins is proof of a physiologically genetically determined
formation of antibodies. A similar explanation can be
offered for the presence of heterohemolysins and
heterohemagglutinins. Another possibility of the
presence of heterohemolysins is the explanation offer-
ed by the presence of Forssman's antigen in various
organisms which may infect the host and cause the
production of Forssman's antibody, a sensitizer for
sheep cells. This antigen may also be present in

absorbed food, which could offer the same possibilities.
The evidence for spontaneous origin of normal hema--
gglutinins and hemolysins is presented by certain re-
gularities in their distribution. Whatever the cause
may be for these substances, no staisfactory answer
as yet has been offered.

Purpose of This Work

This work has had as the paramount purpose, a
serological comparison of this very interesting group
of animals, the Serpentes, to an unrelated biological
group, the Mammalia. Immunologists have confined most
of their work to the Mammalia and the Aves, with little
regard for the lower animal Phyla. The majority of the
work involving the Reptilia has been in regard to the
venoms of the poisonous snakes. This has been a very
practical phase of immunology and a very essential one,
since this field is of commercial importance. With
these facts in mind this work was started, hoping to
add some useful biological information to our meager
knowledge of the relationship of Immunology and Biology.

Initial investigations developed many interesting
lines of study, but in order to conduct a rather
systematic study of this group, certain questions were
definitely decided upon and the work continued in those
directions.

Since the literature contained many papers in regard to blood grouping of lower animals, and with the statement of Snyder concerning the importance of blood grouping in lower animals, before me, the first question to be investigated was, is it possible to devise a blood group classification for the Serpentes, based on the immunological phenomenon of isohemagglutination?

The question of possible serological relationship of the Reptillia and the Mammalia was first attacked by attempting to determine whether snake erythrocytes contained agglutinable factors which could be agglutinated by the alpha and beta agglutinins in human serum. In contrast to this, the next question was to determine whether snake serum contained agglutinins for human erythrocytes, and if so, their relationship to the A and B agglutinogens of human cells.

These two questions are the only ones discussed in this work relative to the possible serological relationship of the Serpentes and the Mammalia. Other phases such as relationship according to the precipitin reaction will be carried out in a later study.

Some of the results noted in the above work led to a study of the cause of the hemolytic effect of fresh snake serum for human cells. Since this action depends upon the presence of a heterohemolysin and a complement, and no reported results could be found

in the literature concerning the presence of these
factors in snake serum, the question arose as to the
possibility of demonstrating these two factors in
snake serum. This is of importance because it is
usually considered that complement is a constituent
of the blood of warm-blooded animals.

Since the material was available, several in-
cidental facts were determined about snake blood.
These are not of special importance and are included
only for additional information concerning this
interesting group of animals.

Experimental Work
I. Hemagglutination Within the Suborder Serpentes

Since the discovery of human blood groups by
Landsteiner in 1901, many attempts have been made to
describe a similar condition in lower animals.

These investigations have been mainly along
three lines: first, to determine whether or not
isohemagglutinins and isohemagglutinogens are present
in various animal species, and if so, the possibility
of devising a grouping plan; second, to determine the
amount of hemagglutination between species; and,
third, investigations concerning the similarity of
demonstratable hemagglutinogens and hemagglutinins
in lower animals to those of man.

The value of such investigations lies along
the following three lines: 1. the study of the problem
of evolutionary development of man could be supplement-
ed if serological relationships could be demonstrated;
2. additional information regarding taxonomic relation-
ship of animals would be available; 3. a study of the
inheritance of these factors would be more possible
than it is in man.

A brief review of the literature along these
lines will show that, although some conflicting
reports are found, much work has been done and that
a definite advance is being made. The reported
results may be divided into four groups: 1. those
investigators reporting the absence of isoagglutin-
ation in lower animals; 2. those demonstrating iso-
agglutination in lower animals but unable to determine
definite blood groups; 3. those who have been able to
demonstrate blood grouping within species; and 4.
those who have demonstrated hemagglutination between
species.

Several investigators represent the first group
who report the absence of isoagglutination in lower
animals. Hektoen 1907 (43) found no isoagglutination
in rabbits, guinea pigs, dogs, horses, and cattle.
Weszecsky 1920 (44) reports negative results in cattle,

rabbits, guinea pigs, and chickens. Rhodenberg 1920
(45) failed to find isoagglutination in rats. McDowell
and Hubbard 1922 (46) found only 2 cases of isoagglu-
tination in 1180 possibilities with mouse blood. Boyd
and Walker 1934 (47) found no individual blood diff-
erences in guinea pigs or mice. Fishbein 1913 (48)
found no isoagglutination in frogs.

Those reporting isoagglutination in various animal
species but unable to formulate definite grouping are
as follows: Ingebregsten 1912 (49) found it impossible
to group cats according to isoagglutination. Fishbein
1913 (48) demonstrated isoagglutination but no group-
ing in swine, cattle, sheep, rabbits, and dogs. Snyder
1924 (50) found no constant isoagglutination in rabbits.
Walsh 1924 (51) could determine no evidence of group-
ing in rabbits. Karshner 1923 (52) demonstrated iso-
agglutination in bovine blood although it occurred
very irregularly. Lawson and Redfield 1930 (53)
found no grouping in guinea pigs or rabbits.

Several investigators have been successful in
determining definite blood groups within species of
lower animals. Newdow 1928 (54) reported the presence
of four groups in horses analogous to the human
groups. Schermer 1929 (55) reported similar results.
Little 1929 (56) identified three principal groups

in bovine blood. Lawson and Redfield 1930 (53) report a possibility of groups in monkeys similar to human groups. Weinert 1933 (57) concludes that anthropoid apes may be divided into the same groups as man. Burghardt 1933 (58) confirmed the grouping of horses into four groups. Herman 1936 (20) grouped 854 out of 910 samples of horse blood into four groups. Gorer 1936 (59) found a definite grouping in mice.

A few workers report on heterohemagglutination or lack of heterohemagglutination between species of lower animals. Kolmer and Matsumoto 1920 (60) found that horse serum contains agglutinins and hemolysins for rabbit cells. McDowell and Hubbard 1922 (46) found agglutinins in guinea pigs and sheep sera for mouse erythrocytes, but rat serum did not agglutinate mouse cells. Walsh 1924 (51) found that guinea pig and horse serum gave constant agglutination of rabbit cells.

Of the reported results discussed above only two investigators have worked with animals lower than mammals in the animal scale. Weszecsky found no evidence of isohemagglutination in chickens, and Fishbein found none in frogs.

If studies of this type are to be of any value in

taxonomy and in the study of evolutionary development, the work must be extended to all possible phyla of the animal kingdom. With this in mind these serological studies of the Reptilia were started.

For convenience of arrangement of this and following work, the suborder Serpentes was selected as the first group of this phylum for study. The first questions to be answered in the study of this group were: Do snake bloods exhibit isoagglutination and can snakes be grouped serologically on this basis?

Due to the difficulty of keeping a number of specimens on hand at one time, the results must be given in two sets. In all cases the technic was the same. Serum was used undiluted and a one per cent suspension of cells made from packed cells. Equal amounts of serum and cell suspension were mixed in an open paraffin ring on a glass slide by rotating on a flat surface. Each set up was allowed 15-20 minutes incubation with intermittent rotation. The readings were made with a compound microscope giving a magnification of 40, and results recorded in terms of amount of agglutination.

Preliminary tests to determine a suitable incubation temperature were carried out. Identical preparations were incubated at $6°C.$, $37°C.$, and room

temperature. Microscopic examination revealed no difference in results with the three temperatures used; therefore, all subsequent tests were done at room temperature.

Table number I represents reactions between species. Table number II shows reactions between individuals.

Cells

Serum	5 Timber Rattle-snake	17 Copper-Head	5 Bull Snake	5 Black Snake	9 Prairie Rattle-snake	1 King Snake	1 Water Snake
5 samples Timber Rattle-snake	-	-	-	-	-	-	5 sera +
17 samples Copperhead	-	-	-	-	-	-	17 sera +
3 samples Bull Snake	1 sera + 2 sera -	-	-	-	1 sera + 2 sera -	-	-
5 samples Black Snake	-	-	-	-	1 sera + 4 sera -	-	-
9 samples Prairie Rattle-snake	-	-	-	-	-	-	5 sera + 4 sera -
1 sample King Snake	-	-	-	-	-	+	-
1 sample Water Snake	-	-	-	-	-	-	-

Table I

Cells	V-9	V-10	V-11	V-7	V-8	G-1	G-2	G-3	XI-1	XII-1	XIII-1	XIV-1	XV-1	XVI-1	XVI-2
Serum V-9	-	-	-	-	-	1+	3+	-	-	-	-	-	-	-	-
V-10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V-11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V-7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
G-2	1+	-	1+	3+	1+	-	-	-	-	1+	±	-	-	1+	1+
G-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
XI-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
XII-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
XIII-1	3+	-	-	4+	3+	-	-	-	-	-	-	-	-	-	-
XIV-1	4+	4+	CH	CH	4+	-	-	-	CH	4+	-	-	-	4+	4+
XV-1	2+	±	3+	4+	4+	-	-	-	3+	3+	-	-	-	3+	3+
XVI-1	-	-	-	-	-	±	±	-	-	-	-	-	-	-	-
XVI-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table II

The cases in which agglutination did occur were irregular; however, the serum of five timber rattlesnakes, seventeen copperheads, and five out of nine prarie rattlesnakes did agglutinate erythrocytes of a water snake Natrix sipedon. The above mentioned sera were from snakes belonging to the family Crotalidae, the water snake to the family Colubridae. These were the only tests made involving these two families together.

From the above tables it is evident that in no case was agglutination found between individuals of the same species, neither was agglutination found between species of the same genera.

In table number II most of the cases of agglutination were found with the sera of two water snakes of the same species, Natrix sipedon pictiventris. Both of these agglutinated or hemolyzed samples of cells from nine snakes of four species and three genera. This indicates that there is heterohemagglutination between genera of the same family, the Colubridae.

Mention must be made of the three cases of hemolysis found in table II. The serum of one specimen of Natrix sipedon pictiventis hemolyzed two of five cell suspensions of Pituophis sayi and one sample of Pituophis mugitas. In all work done these were the

only cases of hemolysins found in snake serum for snake erythrocytes.

The following conclusions may be made in regard to the series of specimens examined in this work, although the use of other species may alter these and make other conclusions possible.

1. Isohemagglutination does not occur in snakes.

2. Hemagglutination between species of the same genera was not found.

3. Heterohemagglutination occurs between genera but rather irregularly.

4. Heterohemagglutination between families was found to be rather constant.

5. Three cases of heterohemolysins were found.

II. The Incidence of Agglutinogens in Snake Erythrocytes for Human Alpha and Beta Agglutinins.

The property of agglutination of heterologous cells by human serum has been very well demonstrated by several investigators. Some of these have been content with establishing this fact alone, while others have gone on to determine what factors in these heterologous cells are responsible for this reaction and to see if these factors are in any way similar to the agglutinogens in human cells.

We find also many workers who report grouping
in animals on the basis of isoagglutination and who
compare this to the grouping in man without stating
whether or not the hemagglutinating factors are
similar to those in man.

Landsteiner and Miller (61) found the isoagglutin-
ogens of anthropoid apes to be identical with those
of human blood, and in lower monkeys a factor similar
to the human isoagglutinogen B was found. Herman (20)
determined a resemblance between horse and human
agglutinogens. Gorer (59) found a fairly close relation
between the agglutinogen factor in mice and the A_1 and
A_2 factors in man. Buchbinder (62) could find no
agglutinogens identical with or closely related to
those in human cells in Macacus rhesus erythrocytes.
Hirano (63) found that the cells of Philippine mon-
keys are agglutinated by some human serum irrespect-
ive of blood groups. Lower (64) concluded that the
agglutinins and agglutinogens in the blood of dogs
are very similar to group B of man.

Since very little information is available con-
cerning members of the animal scale lower than mammals,
this work was done in order to see if the Serpentes
might have agglutinogens in their erythrocytes simi-
lar to the A and B agglutinogens of human erythrocytes,

or if human serum might contain agglutinins for other agglutinable factors in snake erythrocytes.

The technic used for these determinations was to place equal volumes of human sera of each of the four types and one per cent suspension of the snake cells to be studied in open paraffin rings on a glass slide. These were mixed by intermittent rotation of the slide for fifteen to twenty minutes, and then examined microscopically for amounts of agglutination. Preliminary tests at $6^{\circ}C.$, $37^{\circ}C.$ and room temperature showed no difference in amounts of agglutination; therefore, all subsequent tests were done at room temperature.

Human sera of types O, A, and B provided the Alpha and Beta agglutinins to test for A and B agglutinogens in the snake erythrocytes. Type AB serum was used to determine whether any other agglutinogen-agglutinin complex might be found for human serum and snake cells.

In the first series of 465 tests, fifteen specimens of snake cells representing seven species of five genera were set up with ten samples of type O, ten of type A, eight of type B, and three of type AB sera.

Only three cases of agglutination were found in this series. The cells of one of five specimens of the bull snake, Pituophis sayi, were agglutinated by two samples of type O serum and by one sample of type A

serum. These reactions were not complete, being recorded as one plus and two plus agglutination.

A second series of twenty-seven specimens of snake cells representing five species of five genera against ten samples of human sera representing all four known types was carried out in the same manner. Only two cases of weak agglutination could be found in this series, one with cells of a bull snake, Pitouphis sayi, and one with cells of a water snake, Natrix sipedon.

From the above results it is evident that the snake cell samples used in this work do not contain agglutinogens for human Alpha and Beta agglutinins. The inconstancy, in regard to Alpha and Beta factors, of the few positive agglutination reactions found would indicate this. Of the five positive agglutination reactions found, little can be said except that a larger series will be necessary to determine the cause of these results. It may be that some human sera contains agglutinins for snake cell agglutinogens, these probably having no correlation to the A and B factors.

Even though agglutinogens for A and B agglutinins could not be demonstrated by the agglutination reaction, it was thought possible that such factors might exist, yet for some reason agglutination might not occur.

As a further check on the above work, the absorption technic was used.

The titers of a known human type A serum and a known type B serum were determined by using appropriate cells. Each serum was then absorbed with two parts of washed packed snake cells of various species. This absorption was carried out at room temperature with agitation of the mixture for one hour in a shaking machine followed by overnight incubation in the ice box. The titer of the supernatant serum from these mixtures was then determined. Before absorption the type A serum used had a titer of 1-8 with B cells and B serum had a titer of 1-32 with A cells. After absorption both sera had the same titer as in the original titration.

These results are in accord with those previously discussed. It would therefore appear that there is no evidence of agglutinogens in snake cells for human Alpha and Beta agglutinins. There is a possibility of other agglutinin-agglutinogen reactions between human sera and snake cells, but results from a larger series would be necessary before any definite conclusions could be made.

III. Studies of Hemagglutinins in Snake Serum

for Human Erythrocytes

The presence or absence of agglutinins in animal serum for human erythrocytes has been studied by several investigators with the idea in mind of establishing, if possible, some serological relationship between man and lower animals. It has been suggested in another section that the study of evolutionary development of man might be supplemented if serological relationships could be demonstrated. This information is of value only when sufficient evidence has been accumulated concerning the entire animal kingdom. Data in the nature of this work can supplement that furnished by other types of tests as the precipitin reaction.

Williams and Patterson 1918 (65) found that with nineteen samples of horse serum, twelve produced some agglutination of human cells. Kolmer and Matsumoto 1920 (60) found that practically all horse sera examined contained agglutinins for human erythrocytes. Neowdow 1928 (54) and Schermer (55) 1929 reported the presence of four blood groups in horses analogous to human groups. Karshner 1928 (52) found that bovine and chicken sera consistently agglutinated human cells. Hirano 1932 (66) concluded that the anti A agglutinin serum of Phillipine monkeys is

different from the anti A agglutinin in human serum, but monkey serum can be used in place of type B human serum for routine typing. Buchbinder 1933 (67) found that Macacus rhesus sera contained a hemagglutinin which was indistinguishable from alpha isoagglutinin of human serum. Braido 1933 (68) found that normal sera of sheep, ox, and horse agglutinate human erythrocytes, each differently, and differently from human serum. Stuart, Sawin, Wheeler and Battey 1936 (69) established the presence of group specific agglutinins in rabbit serum for human cells, finding that 39% of the rabbit sera used, contained specific agglutinins for A cells, 16% specific agglutinins for B cells, and 38% failed to agglutinate any type of human cells. Herman 1936 (20) found a resemblance between horse and human agglutinins.

Of the above papers reporting agglutination of human cells by animal sera, only one reports the use of serum from animals lower in the zoological scale than Mammals. Karshner found that chicken serum constantly agglutinated human cells.

Since it had been found in previous work that snake serum exhibited various degrees of hemolysis or agglutination of human cells, it seemed desirable to make a more complete study of this to determine the distribution in snakes of this hemagglutinating

property.

Preliminary work showed that freshly drawn snake serum hemolyzed human cells, but that old serum or serum inactivated at 56°C. for fifteen minutes did not. In all of the following work snake serum which exhibited this lytic action was inactivated before use.

The technic used was to place equal volumes of undiluted snake serum and a one percent suspension of human cells in an open paraffin ring on a glass slide. These were mixed by intermittent rotation of the slide for fifteen to twenty minutes, and then examined microscopically for amounts of agglutination. Preliminary tests at 6°C., 37° C. and room temperature showed no difference in amounts of agglutination, therefore all of the following work was carried on at room temperature.

A total of 112 samples of snake serum representing ten genera and eighteen species were set up with at least ten samples of human cells, representing all four known types. Of these 112 samples thirty-five (31.2%) did not agglutinate human cells, two (1.8%) agglutinated A and AB cells, twenty-two (19.6%) agglutinated A, B, and AB cells, thirty-seven (33%) agglutinated all four types of human cells and sixteen (14.4%) gave irregular results.

No correlation with genus or species could be determined from these results. As an illustration it was found that some samples of serum from timber rattlesnakes did not agglutinate human cells, others agglutinated A and AB cells and others agglutinated A, B, and AB cells.

Since it was demonstrated that a difference did exist in the agglutinating property of snake sera for human cells, the next question to be answered was: Is this agglutination due to specific agglutinins similar to the agglutinins in human serum for human cells?

The first serum chosen for this study was that of a Bull snake, Pituophis sayi. This specimen of serum when mixed with 100 samples of human blood, which had previously been typed with known human serum, gave the following results: Forty-eight type O samples were not agglutinated, but thirty-seven type A, eleven type B and four type AB samples were agglutinated. The amounts of agglutination of types A, B, and AB cells varied from two plus to four plus.

Four portions of this inactivated serum were then absorbed with washed packed cells of the four human types. This absorption was carried out by mixing one part of undiluted serum with two parts of packed cells in a shaking machine for one hour. The four samples of

centrifuged supernatant serum were then set up with the one per cent cell suspensions used in the preliminary determinations. The results obtained are given in table III.

	48 specimens type O cells	37 specimens type A cells	11 specimens type B cells	4 specimens type AB cells
Inactivated Serum	-	+	+	+
Inactivated Serum absorbed with O cells	-	+	+	+
Inactivated Serum absorbed with A cells	-	-	+	+
Inactivated Serum absorbed with B cells	-	+	-	+
Inactivated Serum absorbed with AB cells	-	-	-	-

Table III

The results given in the above table indicate that the agglutinating property of this snake serum for human cells is due to specific agglutinins for agglutinogens A and B in human cells. These agglutinins were absorbed specifically by the corresponding agglutinogen and also were removed by absorption with AB cells. This would indicate a similarity of agglutinins in snake and human sera. Additional work will be necessary to determine whether these factors are identical.

Similar determinations were made with the serum of a Diamondback Rattlesnake and serum of a Timber Rattlesnake. Results identical to those given above were ob-

tained with these two sera.

In the survey of the distribution of hemagglutination by snake serum, it had been found that thirty-three per cent of the snake sera examined agglutinated type O cells in addition to A, B, and AB cells. Since the three sera used in the above work exhibited specific absorbable agglutinins for human A and B agglutinogens, the question arose as to what factor might be responsible for the agglutination of type O cells, which do not contain A and B agglutinogens.

For this determination two sera were selected which agglutinated cells of all four types. Portions of these sera were absorbed with each type of human cells, and the centrifuged supernatant serum set up with samples of O, A, B, and AB cells. Identical results were obtained with these two sera and are given in table IV.

	Type O cells	Type A cells	Type B cells	Type AB cells
Inactivated Serum	+	+	+	+
Inactivated Serum absorbed with O cells	-	+	+	+
Inactivated Serum absorbed with A cells	-	-	+	+
Inactivated Serum absorbed with B cells	-	+	-	+
Inactivated Serum absorbed with AB cells	-	-	-	-

Table IV

The above results show that the agglutinating property of these sera for type O cells was removed by absorption with any type of human cells. This would indicate that some snake sera contains an agglutinin for all human cells. This may be considered as a species agglutinin.

The two sera which agglutinated only A and AB cells were absorbed with A cells and with B cells by the previously described technic. Serum absorbed with type A cells would not agglutinate either A or AB cells, but serum absorbed with B cells retained its agglutinating property for A and AB cells. This must mean that an agglutinin similar to the Alpha agglutinin is present in these two sera.

To demonstrate the possibility of determining unknown human blood types with snake serum, two portions of each of five sera were absorbed with type A cells and type B cells respectively. These absorbed sera were set up with twenty-five samples of unknown human cell suspensions, by the usual technic. Following this, each cell sample was typed by routine procedure for verification of the type. The results with snake serum are given in table V.

|	Serum absorbed with A cells	Serum absorbed with B cells	
Patient's cells	-	-	Type O
Patient's cells	-	+	Type A
Patient's cells	+	-	Type B
Patient's cells	+	+	Type AB

Table V

The results on the above table show that, by appropriately absorbing a selected snake serum, a determination of human blood types is possible. Type O cells will not be agglutinated by absorbed serum. Type A cells will be agglutinated by serum absorbed with B cells, but not with serum absorbed with A cells. Type B cells will be agglutinated by serum absorbed with A cells, but not by serum absorbed with B cells. Type AB cells will be agglutinated by serum absorbed with either A or B cells.

Since it had been found thatthe hemolytic property of snake serum for human cells could be removed by inactivation, the question arose as to the stability of the agglutinating property. For this determination, serum from five Bull Snakes was inactivated in an oil bath for fifteen minutes at temperatures of 45°, 50°, 55°, 60°, 65°, and 70°Centigrade. Following the heating each serum was diluted serially from 1-2 to 1-128.

Equal volumes of these serum dilutions and suspensions of each type of human cells were mixed as previously described. The amount of agglutination was determined for each dilution to show the drop in titer due to inactivation.

Table VI shows the results obtained with one of these sera. Some variation was found for the five sera, but as a rule the agglutinins for A, B, and AB cells were removed at 65° or 70°. The removal of agglutinins for type O cells was more variable, but the majority were removed at 50°C. and 55°C.

These results compare very favorably with the thermostability of human isoagglutinins.

VI
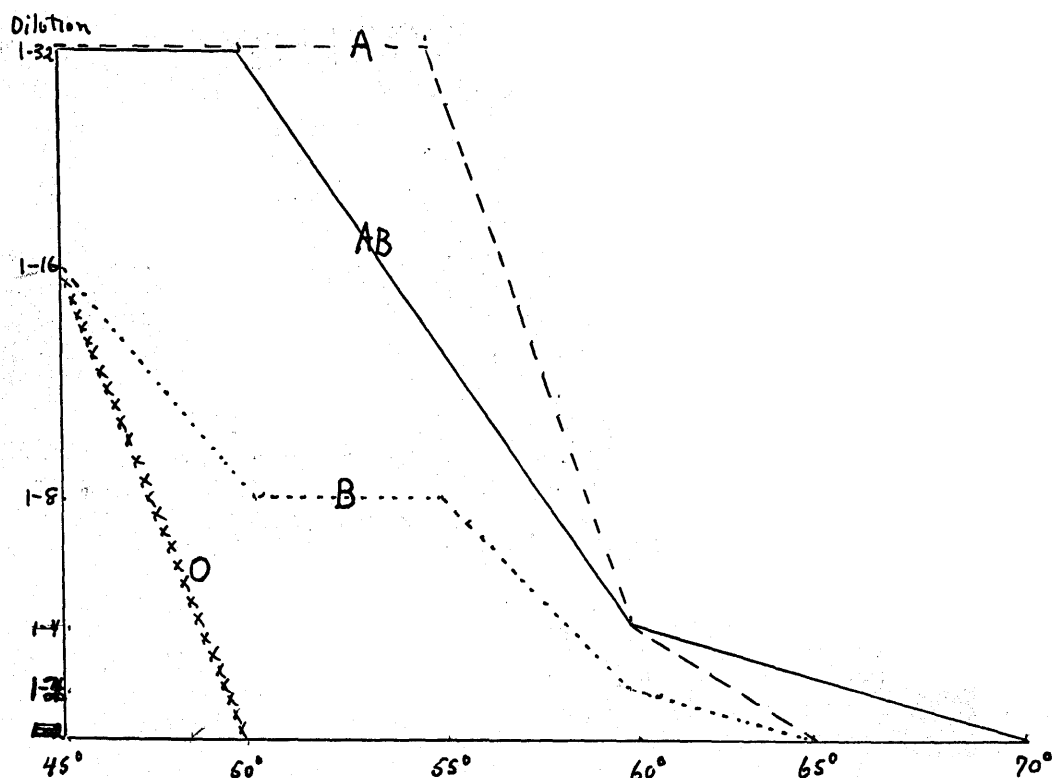
Summary

In a study of the agglutinating property of 112 snake sera for human cells, it was found that 31.2% did not agglutinate human cells, 1.8% agglutinated A and AB cells, 19.6% agglutinated A, B, and AB cells, 33% agglutinated all four types of human cells and 14.4% gave irregular results.

These 112 samples represent only eighteen species from ten genera, so consequently the results do not gove a complete picture of the entire suborder Serpentes. Nevertheless it is a large enough representation to be of significance in demonstrating the fact that a serological similarity does exist between the serum of man and certain reptiles.

Not only do these sera agglutinate human cells, but the agglutinating factors for A, B, and AB cells are specifically absorbed out by A and B human cells. The agglutinmfor type O cells which is found in some snake sera may be regarded as a specie agglutinin since it is absorbed with any type of human cells. Since these agglutinating factors can be specifically absorbed, it is possible to use appropriately selected and absorbed snake serum for determination of human blood types. This procedure is not offered as a practical method of blood typing, but to add information to our meager knowledge concerning the serological relationship

of lower animals and man. It is hoped that additional
work may soon be carried out with sera representing other
species, genera and families and that ultimately more
complete conclusions can be made in regard to distri-
bution of these agglutinins in snakes.

Conclusions

1. Snake serum may or may not contain agglutinins
   for human cells.

2. Agglutinins are found in some snake sera for
   human A, B, and AB cells. They may be
   specifically absorbed by human A and B cells.

3. An agglutinin for O cells was observed in cer-
   tain snake sera. It may be considered as a
   species agglutinin since it is completely ab-
   sorbed by any type of human cells.

4. Appropriately absorbed snake sera may be used
   to determine human blood types.

5. Snake and human hemagglutinins are equally
   thermostable.

IV. Studies on the Hemolytic Property of Snake Serum

The hemolytic property of normal animal serum for heterologous red cells has been known for some time. This property is usually explained as being due to the presence of a natural occuring sensitizer and the complement of the fresh serum.

It has been mentioned in a previous section that fresh snake serum exhibits a lytic action on human red cells. Subsequent work has shown that snake serum is also quite frequently hemolytic for sheep, guinea pig, and rabbit erythrocytes.

A rather careful serarch of the literature failed to disclose any published experimental investigations of the heterohemolysins present in snake serum. It seemed therefore desirable to make such an investigation. The present paper represents a report of experiments designed to answer, in so far as possible, certain fundamental questions bearing upon these heterohemolysins. These questions may be stated briefly as follows.

1. What is the incidence and titer of the anti-sheep and anti-human heterohemolysins in the samples of snake serum available?

2. Will snake serum diluted beyond its natural hemolytic titer function as a complement to bring about

hemolysis of red cells sensitized by antibody obtained from immune rabbit serum?

3. Is it possible to restore the hemolytic action to a snake serum which has been inactivated by heat?

4. Is it possible to definitely demonstrate a heterohemolysin (sensitizer) in snake serum and if present, can it be separated from the complement which is present in the fresh serum?

5. How does the complement titer of snake serum compare with that of guinea pig complement and how does it vary in different snakes?

6. How does snake complement compare with guinea pig complement in regard to heat lability and to the effect of storage at different temperatures?

7. What is the anticomplementary titer of normal animal sera, and normal and syphilitic human sera for snake complement?

8. Can snake complement be used successfully in; (a) Bacterial complement fixation? (b) The Wasserman reaction?

A. Incidence and Titer of the Heterohemolysins in Snake Serum.

Thirty-eight specimens of fresh snake serum, representing 13 species of 8 genera caused complete hemolysis of an equal volume of a 1% suspension of

human and sheep cells respectively.

The degree of hemolytic activity of the samples examined varied considerable, with little relationship as to species. This degree of hemolytic activity can best be expressed in terms of .5cc. of the highest dilution of serum necessary to hemolyze .5cc. of a 1% supension of red cells. Various dilutions of the fresh serum were made and mixed with an equal volume of the 1% suspension of red cells. The highest dilution of the serum causing complete hemolysis of the cells was called the titer of the serum.

The average hemolytic titer of the sera for a unit of human red cells was .5cc. of a 1-12 dilution; and for a unit of sheep cells .5cc. of a 1-32 dilution. In comparing the activity of fresh snake serum on human and sheep cells it was found that seventy-five percent of the sera required more serum to hemolyze a unit of human cells than a unit of sheep cells. Twenty-five percent required the same amount.

B. Complementary Action of Snake Serum on Sensitized
   and Normal Cells.

The fact that this hemolytic property was character-istic of all fresh snake serum but not of inactivated serum, suggested the presence of a complement in snake serum. To demonstrate this complement, if it did exist, it would be necessary that it be active in a higher

dilution than the serum dilution which was hemolytic for red cells. With this in mind the following work was carried out.

Serial dilutions of several snake sera were set up with sheep cells sensitized with specific rabbit anti-sheep hemolysin and a similar set of dilutions with sheep cells not treated with the rabbit anti-sheep hemolysin. The highest dilution of the serum causing complete hemolysis of an equal volume of cells was called the titer.

The results obtained show that every sera examined hemolyzed the cells sensitized with the specific rabbit anti-sheep hemolysin to a higher dilution than the cells not treated with the immune serum. Two of these sera showed a very marked difference. These two hemolyzed the untreated cells to a 1-4 dilution and the treated cells to a 1-128 dilution.

These results suggest that a complement, active on cells sensitized with a hemolysin prepared in a warm-blooded animal, is present in snake serum. This complement is active to a higher dilution than the normal hemolytic action of the serum.

C. Reactivation of Heated Snake Serum

To demonstrate further that this hemolytic property of snake serum is due to a hemolysin-complement complex,

attempts were made to reactivate heated snake serum with fresh snake serum.

The snake serum used as a source of complement had a complement titer of .2cc. of a 1-20 dilution. This serum caused hemolysis of normal cells in a 1-4 dilution but not in a 1-12 dilution. The inactivated snake serum originally had a hemolytic titer of 1-32 but after inactivation did not cause hemolysis of red cells even in a 1-2 dilution.

Serial dilutions, beginning with a 1-12 dilution of the fresh snake serum were made and .5cc. of each of these dilutions was added to a series of tubes which had received .5cc. of 1% cells and .5cc. of heated snake serum diluted 1-5.

It was found that hemolysis of the cells occurred to a 1-64 dilution of the fresh snake serum. This shows that the inactivated serum had sensitized the red cells so that they would be hemolyzed by a dilution of the fresh serum which was above its hemolytic titer but within its complement titer.

It should be mentioned that the hemolytic titer of fresh snake serum seems to depend upon the titer of the sensitizer rather than the titer of the complement. Results indicate that invariably the complement is active to a higher dilution than the sensitizer.

Since it had been possible to reactivate heated snake serum with fresh snake serum, the question arose as to the possibility of using guinea pig complement in the same way.

Snake sera with high normal hemolytic titers were inactivated at 56^{0}C. for 15 minutes. A unit of guinea pig complement was determined according to Kolmer. To .5cc. of the heated snake serum and 1 unit of cells was added increasing amounts of guinea pig complement from 1 unit to 8 units. In no case did hemolysis occur.

Further work will be necessary to determine the cause of this difference between snake and guinea pig complement in reactivating heated snake serum.

D. Separation of the Complement and the Heterohemolysin

Although the above work has demonstrated the presence of a complement and a hemolysin in snake serum, it seemed desirable to adopt another experimental procedure to separate and to demonstrate these two factors.

To separate these two factors, a procedure was used which will demonstrate the fact that guinea pig complement is inactive at 0^{0}C., and that red cells will absorb hemolysin but not complement from a mixture of hemolysin and complement when kept at 0^{0}C.

Fresh snake serum which had a normal hemolytic titer of 1-32 was diluted 1-4. Five tenths cc. of this serum dilution, .5cc. of a 1% cell suspension, and 2cc. of isotonic saline were thoroughly chilled in a brine

bath maintained at 0°C. These materials were mixed and replaced in the 0°C bath. A similar tube was mixed and placed in a 37°C. water bath. These tubes were allowed to stand for 30 minutes after which the tube in the 37°C. bath showed complete hemolysis and the tube in the freezing bath no hemolysis. The tube showing no hemolysis was packed in chilled brine and rapidly centrifuged. The clear supernatant fluid was separated from the sediment and these returned to the brine bath immediately.

The supernatant fluid removed from the mixture was tested for the presence of complement and for its hemolytic action on normal red cells. The following results were obtained.

1. Supernatant fluid .5cc. + Saline .5cc. + Cells .5cc. = No hemolysis.

2. Supernatant fluid .5cc + Heated snake serum .5cc + Cells .5cc. = Complete hemolysis.

3. Saline .5cc. + Heated snake serum .5cc + Cells .5cc. = No hemolysis.

These results show very definitely the presence of complement and a thermostable hemolysin in fresh snake serum.

The sediment of the above experiment was brought to a 1.5cc. volume with saline and allowed to stand at room temperature for 30 minutes. Invariably

hemolysis of the cells resulted.

The latter result is an anomolous one and needs further investigation since it is not comparable to the result obtained when using a prepared hemolysin, guinea pig complement, and sheep cells.

Two explanations which might be offered to explain the result obtained with the sediment are ; First, Although snake complement does not produce hemolysis of sensitized cells at $0^{\circ}C.$, a portion of the complement may be bound by the sensitized cells and become active when brought to a higher temperature. Second, There may be present some hemolytic agent which does not depend upon complement for its activity.

E. Complement Titer of Various Snake Sera.

Since it had been shown that a complement action was exhibited by snake serum, it seemed desirable to compare the activity of these sera to that of guinea pig complement by routine complement titration according to Kolmer.

The rabbit anti-sheep hemolysin used in the previous work had been titrated in an excess of guinea pig complement, therefore was retitrated in an excess of snake complement. In no case was this amount hemolytic for normal sheep cells. The unit of hemolysin was found to be the same as that found with guinea pig complement.

In each of the following routine complement titrations, duplicate determinations were made with normal unsensitized sheep cells to rule out the activity of normal hemolysins.

Fifteen samples of sera, representing four species of snakes, were titrated. The average complement titer for this series of snake sera was .2cc of a 1-20 dilution. The highest titer found was a .1cc. of a 1-20 dilution and the lowest .25cc. of a 1-20 dilution. In no case was .5cc of a 1-20 dilution hemolytic for one unit of unsensitized cells in a 3cc. total volume.

The results obtained from these titrations would indicate that complement is present in snake serum in amounts comparable to that found in high titered samples of guinea pig serum. They would also indicate that the average complement titers for snake serum is greater than the average for individual samples of guinea pig serum, which vary in complement content quite markedly.

To determine the constancy of the complement titer of individual specimens, bleedings were made at irregular intervals and the titer determined. Typical results are those of a water snake, Natrix rhombifera.

1. January 19 1 unit .2cc. of a 1-20 dilution
2. February 20   "   .2cc.   "   "
3. April   3   "   .15cc.   "   "

4. April   9   1 unit   .2cc. of a 1-20 dilution
5. April   22   "   .2cc.   "   ."
6. April   27   "   .15cc.   "   "

This would indicate that the complement in snake serum is a very constant constituent, varying only slightly from week to week.

F. The Effect of Heat and Storage on Snake Complement

To further compare this complement to guinea pig complement, the lability and rate of deterioration was determined.

In a previous section it was stated that fresh snake serum or serum kept frozen for several days, hemolyzed human cells, but that serum kept for a longer period or heated to 56^{0}C. for 15 minutes did not exhibit this action. The following work was done to determine exactly the rate of destruction and deterioration of this complement.

Specimens of fresh snake serum were placed in a 56^{0}C. water bath and a sample removed every minute for ten minutes. These samples were then titrated against normal and sensitized cells. All of the specimens examined showed a loss of hemolytic action after 5 or 6 minutes heating.

These results are comparable to the inactivation of the complement of warm-blooded animals.

To determine the rate of deterioration of snake complement, portions of identical serum were stored at temperatures of $0°C.$, $6°C.$, $20°C.$, and $37°C.$ Routine complement titrations were carried out at irregular intervals to determine the existing titer of the serum.

	$0°C.$	$6°C.$	$20°C.$	$37°C.$
Fresh	0.15 cc. (1-20)	0.15 cc. (1-20)	0.15 cc. (1-20)	0.15 cc. (1-20)
1 day	0.15 cc. (1-20)	0.2 cc. (1-20)	0.2 cc. (1-20)	----
3 days	0.2 cc. (1-20)	0.25 cc. (1-20)	0.4 cc. (1-20)	
5 days		0.35 cc. (1-20)	0.35 cc. (1-10)	
7 days	0.25 cc. (1-20)	0.4 cc. (1-20)	----	
10 days		0.5 cc. (1-20)		
16 days	0.4 cc. (1-10)	0.5 cc. (1-10)		
18 days	----	----		

Table VII

The results obtained with the serum of a water snake are very typical of the sera examined, and are given in Table VII. This serum originally had a complement titer of .15cc. of a 1-20 dilution. The portions stored at 0°C. dropped in titer to .4cc. of a 1-10 dilution in 16 days and disappeared in 18 days. At 6°C. the titer dropped to .5cc. of a 1-10 dilution in 16 days and disappeared in 18 days. At 20 C. the titer dropped to .35cc. of a 1-10 dilution in 5 days and had disappeared by the seventh day. At 37°C. the complement had deteriorated in 24 hours.

This rapid decrease and loss of titer at high temperatures and the slower decrease in titer at low temperatures, compares very favorably to the deterioration of guinea pig complement.

G. Anticomplementary Action of Normal Animal Sera

Since this preliminary work demonstrated the presence of a complement in snake serum, the question arose as to the possibility of its use in complement fixation tests.

This complement had been found to be active in the presence of rabbit serum, and as preliminary check to complement fixation tests it seemed desirable to determine its activity in the presence of other animal sera.

Anticomplementary titrations were done with several samples of normal animal sera with the following results.

5 Normal Guinea pig sera-not anticomplementary in a 1-2
dilution

3 " Rabbit	"	"	"		"	"
Pooled Beef	"	"	"		"	"
4 Normal Human	"	Average anticomplementary titer 1-128				
4 Syphilitic Human	"	"	"			1-4
Pooled Hog	"	"	"			1-8
" Sheep	"	"	"			1-2
" Horse	"	"	"			1-64

The above results suggest that complement fixation tests using rabbit, beef or guinea pig antiserum could be carried out, but the use of snake serum as complement in the presence of human serum would depend upon the anticomplementary titer of the serum used.

The difference in anticomplementary titers observed between normal and syphilitic human sera will bear further investigation. This difference may be of signifance in differentiating these sera, but it will be necessary to examine a larger series of both types of serum before any definite conclusions can be made.

H. Complement Fixation

1. Bacterial Complement Fixation

Since the previous work showed that rabbit serum

was not usually anticomplementary for snake complement, a rabbit antiserum was prepared against Salmonella enteritidis for the bacterial complement fixation tests. The antigen was a saline suspension of the enteritidis organisms.

The unit of antigen to be used in the routine tests was determined by hemolytic, anticomplementary and antigenic titrations. In the latter two, snake serum was used as complement. Two and one-half units of the antigen were used in the final tests, and set ups made as given in Table VIII.

No.	Anti-gen 2.5 units	Anti-serum 1-10	Normal Rabbit Serum	Comple-ment 2.0 (Snake)	Sal-ine	Hemo-lysin 2 u.	Cells 1 u.	Results
1	.5 cc.	.5 cc.	--	.5 cc.	.5 cc.	.5cc.	.5cc.	No Hemo-lysis
2	.5 cc.	--	--	.5 cc.	1.0 cc.	.5cc.	.5cc.	Com-plete Hem.
3	--	.5 cc.	--	.5 cc.	1.0 cc.	.5cc.	.5cc.	"
4	.5 cc.	--	.5 cc.	.5 cc.	.5 cc.	.5cc.	.5cc.	"
5	--	--	.5 cc.	.5 cc.	1.0 cc.	.5cc.	.5cc.	"
6	--	--	--	.5 cc.	2.0 cc.	--	.5cc.	No Hem.
7	--	--	--	.5 cc.	1.5 cc.	.5cc.	.5cc.	Com-plete Hem.
8	--	--	--	--	2.0 cc.	.5cc.	.5cc.	No Hem.

Table VIII

The above results show that in tube 1 containing the specific antigen antibody complex, two full units of complement were bound. This was a specific fixation as the complement was not fixed in control tubes 2, 3, 4, and 5. The snake serum was not hemolytic for red cells in the dilution used as indicated by tube 6.

This shows that snake serum can be used as a source of complement in a routine bacterial complement fixation test, when rabbit antiserum is used.

2. Complement Fixation with Kolmer Antigen

Although it had been found that normal human serum was anticomplementary to a rather high dilution, there was a possibility that complement fixation could be demonstrated with syphilitic human serum and Kolmer antigen. The anticomplementary titers of 4 syphilitic sera had been found to be 1-2, 1-4, 1-8, and 1-4. The average titer being 1-4.

The following procedure was used to demonstrate the binding od snake complement by Kolmer antigen-syphilitic serum complex.

No.	Serum 10 u. Kolmer antigen	Comp. 25 f.u.	Saline	Sen. Cells	Results Sera	Results - Sera
1	.5 cc. 1-2	--	.5cc.	1cc.	1cc.	No Hem.	No Hem
2	.5 cc. 1-4	--	"	"	"	Partial Hem.	"
3	.5 cc. 1-8	--	"	"	"	Comp. Hem.	"
4	.5 cc. 1-16	--	"	"	"	"	"
5	.5 cc. 1-32	--	"	"	"	"	"
6	.5 cc. 1-64	--	"	"	"	"	"
7	.5 cc. 1-128	--	"	"	"	"	"
8	.5cc. 1-256	--	"	"	"	"	Partial Hem.
9	.5 cc. 1-2	.5cc.	"	.5cc.	"	No Hem.	No Hem.
10	.5 cc. 1-4	"	"	"	"	"	"
11	.5 cc. 1-8	"	"	"	"	"	"
12	.5 cc. 1-16	"	"	"	"	"	"
13	.5 cc. 1-32	"	"	"	"	"	"
14	.5 cc. 1-64	"	"	"	"	"	"
15	.5 cc. 1-128	"	"	"	"	"	"
16	.5 cc. 1-256	"	"	"	"	"	"

Table IX

Two series of serial dilutions, beginning with a
1-2 dilution, were made for each serum studied. One
series received 10 units of Kolmer antigen while the
other series served as serum anticomplementary controls.
Each tube of both series then received two full units
of snake complement. Overnight ice box incubation was
used for primary fixation. After 15 hours, hemolysin
and red cells as recommended by Kolmer were added.
Secondary incubation was 1 hour at 37°C.

The results, according to Table IX, show that the
lower dilutions were anticomplementary but in the
dilutions comparable to those used in a routine
quantitative Kolmer, two full units of snake somple-
ment were bound in the presence of syphilitic serum
alone. The amount of Kolmer antigen used was not
anticomplementary.

In view of the fact that the normal human sera
examined were highly anticomplementary for snake
complement, this complement could not be used in a
routine Kolmer, but the above work does demonstrate
the similarity of this complement to guinea pig
complement in one respect. It is bound by a Kolmer
antigen-human syphilitic serum complex.

I. Discussion

The above work was designed to demonstrate the

presence of complement and a normal heterohemolysin in fresh snake serum. In addition it was designed to compare in several ways the activity of this complement to the complement of guinea pig serum.

From the results obtained, the hemolytic property of fresh snake serum is due to the presence of a thermolabile complement and a natural occuring thermostable heterohemolysin or sensitizer.

This complement from the blood of a cold-blooded animal compares very favorably in many respects to the complement from warm-blooded animals. In fact from the above results the two appear to be fundamentally identical in their action with possibly two exceptions. 1. It is possible that snake complement may be partially bound by sensitized cells at $0^{9}C.$; 2. Snake complement will reactivate heated snake serum but guinea pig complement will not. These two points bear further investigation.

It is of importance to mention again the difference in anticomplementary titers of normal and syphilitic human sera for snake complement. The full significance of these results can be determined only when a greater number of both sera have been examined.

It seems desirable to discuss here some observations made in connection with part III of the experimental work. In studying the agglutination of human cells by

inactivated snake serum, it was found that 31.2% of 112 samples of snake sera did not agglutinate any type of human cells. In contrast to this it was found with this series of 38 snakes, none of which were used in the first series of 112, that all types of human red cells were hemolyzed by fresh snake serum. The question arose as to the relationship of the hemagglutinins and the hemolytic sensitizer found in snake serum.

Data which had been obtained with 5 samples of sera used in the first series, showed that it was characteristic for all of these samples to hemolyze all four types of human cells but gave variable results in regard to agglutination. Two samples agglutinated A, B, and AB cells, two others agglutinated only A and AB cells, and the fifth did not agglutinate any type of human cells. This, along with the absorption experiments, would indicate that the above mentioned hemolysin (sensitizer) and the hemagglutinins for human erythrocytes are not identical substances. This point warrants further consideration.

J. Conclusions:

The above results warrant the following conclusions.

1. Thirty-eight specimens of fresh snake sera exhibited a lytic effect on sheep and human red cells.

2. Snake serum contains a thermolabile complement and a thermostable heterohemolysin.

3. Snake complement compares very favorable to guinea pig complement in that;

1. It is of a high titer.

2. It is of a constant titer.

3. It is specifically bound in bacterial complement fixation.

4. It is specifically bound by a Kolmer antigen-syphilitic serum complex.

5. It is destroyed in 5 to 6 minutes at $56^{\circ}C$.

6. It deteriorated on standing, rapidly at high temperatures and more slowly at low temperatures.

4. Some normal animal sera are highly anticomplementary for snake complement.

5. This complement from cold-blooded animals will act on cells sensitized with hemolysin prepared in a warm-blooded animal.

6. Heated snake serum can be reactivated by fresh snake complement but not by guinea pig complement.

7. The anticomplementary titer of 4 syphilitic human sera varied from 1-2 to 1-8. The anticomplementary titer of 4 normal human sera varied from 1-64 to 1-256. Whether this is of any signifance or an accidental variation remains to be determined.

8. The apparant partial binding of snake complement at 0^0C. by sensitized cells, bears further investigation.

9. The hemolytic titer of fresh snake serum depends upon the titer of the sensitizer rather than the titer of the complement, since the complement is active to a higher dilution than the sensitizer.

V. Miscellaneous Characteristics of Snake Blood.

    A. Serum-Cell Ratio

It was noticed that when separating the serum from the cells of the first few samples of snake blood obtained, that the amount of serum seemed to be much greater than the amount of packed cells in the bottom of the tube. Since this was so pronounced as compared to the usual proportion of serum and cells for human, rabbit and guinea pig blood, it was decided to determine the ratio of these two fractions of snake blood.

The series examined included forty snakes representing seven species. These snakes had had access to water at all times prior to bleeding. The ratio of these two factors was determined by two methods. 1. By measuring the amount of blood in the sample and then measuring the amount of serum obtained after centrifuging. This could be done only when the blood was secured rapidly enough to measure it before it clotted. 2. The blood was allowed to clot, centrifuged and the heighth of the layer of cells and the heighth of the layer of serum was measured in a tube of uniform size.

The results are expressed in the following table, giving the average determination of the percentage of serum and cells for the stated number of samples.

			Serum %	Cells %
Timber Rattlesnake	5	specimens	70.5	29.5
Copperhead	17	"	73.41	26.59
Bull Snake	3	"	75.45	24.55
Black Snake	5	"	71.67	28.33
Prairie Rattlesnake	8	"	75.93	24.07
King Snake	1	"	76.27	23.73
Water Snake	1	"	74.28	25.72
Total Average	40	"	73.93	26.07

These results show that a marked difference does exist in the serum-cell ratio of snake and mammalian bloods. In man the ratio is usually serum 45% and cells 55% by volume. It is needless to say that the high percentage of serum obtained was very gratifying, since the majority of the experimental work was done with the serum rather than the cells.

B. Sedimentation Rate of Snake Erythrocytes

It was noticed that when snake blood was allowed to stand after defibrination or before clotting occurred, that the cells settled to the bottom of the tube quite rapidly.

Since determination of the sedimentation rate for man had become of clinical importance, and because several samples of blood allowed to stand had serum clots rather than whole blood clots, the following determinations were made.

Two methods were employed, the first is not of much importance in regard to sedimentation rate but is significant as a comparative study of the specific gravity of the two types of cells. One percent suspensions of the packed cells were made in saline and allowed to stand at room temperature. The amount of sedimentation was measured every fifteen minutes. It was found that the snake cells settled out about four times as fast as human cells when made up in a one percent suspension.

The second method is of more significance and was carried out with whole defibrinated blood. Readings were taken every fifteen minutes for one hour and then after two and three hours respectively. The accompanying chart illustrates the results obtained with several samples of various species of blood.
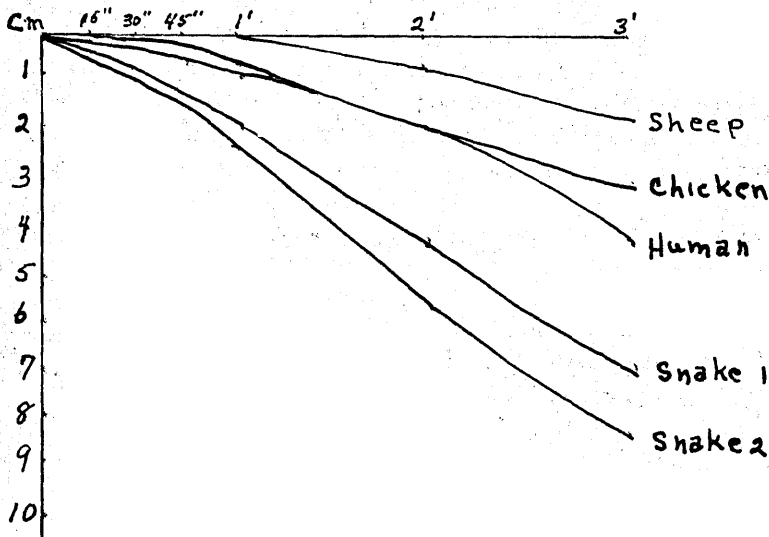
C. Clotting Time

This information cannot be given in any specific manner. No data was kept as to the time involved in the clotting process, so only some of the observations which were made will be given here.

As has been previously stated, a fresh sample of blood allowed to stand without defibrination, may show a settling of the cells and the clotting of the serum. Such samples many times would stand an hour or longer before clotting.

Defibrination could be accomplished but usually required a period of time varying from ten minutes to forty minutes. A difference was noticed in blood obtained from a snake not previously bled or one which had not been bled for some time. Invariably the samples clotted more rapidly from these snakes.

These observations suggest that some very interesting experiments could be carried out which might clarify the mechanism involved in the process of blood clotting. No attempts were made to do any work of this nature. It can simply be said that the clotting time for snake serum is much longer than for the blood of the usual laboratory animals and man.

Discussion

As has been previously stated, the primary purpose
of this experimental work, was to study the possible
serological relationship of the two animal phyla, the
Reptilia and the Mammalia. By such a study it was hoped
that our very meager knowledge of such relationship
would be extended, and also suggest new lines of en-
deavor for future work.

A study of this nature can be pursued along at
least three lines of investigation. 1. A study of
relationship by means of antigenic serum proteins. 2.
A study of relationship by means of the presence or ab-
sence of specific biochemical substances as complement,
which has been proven to be an inheritable factor. 3.
A study of relationship by means of the presence or ab-
sence of circulating normal antibodies which have proven
to be specific inheritable factors.

The first suggested line of investigation, that
by means of antigenic serum proteins, brings immediate-
ly to mind the work of Nuttall and others, but especially
the enormous systematic survey of Nuttall.

In a survey of this nature several things must be
considered in order to evaluate the results obtained.
One must assume that the serum proteins, utilized as the
antigenic substances in preparation of immune sera, are
quite constant in individuals of the same species. This
has been quite extensively investigated in man and found

to be true. This statement can apply only to normal, healthy adult individuals, for under abnormal conditions some type of serum protein may be almost lacking but the total protein remaining nearly normal due to a rise of the other constituents. However under most pathological conditions there is simply a varying of levels, not of chemical make up. It has been found also that the normal protein level is not reached for several days, 12-16, after birth.

Since these factors have been found to be quite uniform from individual to individual, the question of origin is one of prime importance. This question has never been satisfactorily answered although several hypothesis have been advanced. It has been suggested that the source of serum proteins is, 1. disintegrating blood cells, 2. General tissue cells, 3. Bone marrow and 4. the liver.

Nuttall and others, utilizing prepared immune sera against representative members of various animal groups, have demonstrated, by means of the precipitin reaction, the fact that animals which are zoologically related also show serological relationship. The closer the zoological relationship of the animals the greater will be the amount of cross reaction between the animal serum. These results then mean that the biochemical structure of zoologically related animals is similar. The delicate immunological tests used have made it possible to determine this to a finer degree than any chemical test which

can be employed.

Other evidence to be offered in favor of the
relationship of animals by comparing the biochemical
and anatomical structures is the fact that embryologically
the development of the protein content of the plasma,
especially worked out on the chick, shows a change from
a relatively simple structure to one of colloidal com-
plexity in the older embryo. That embryonic develop-
ment proceeds in the same order as evolutionary develop-
ment seems established. Thus the results of immunological
relationship compares very favorably to the anatomical
relationship.

The second line of investigation, which was suggest-
ed is that of determining the presence or absence of
definitely proven inheutable substances associated with
the serum proteins. The factor in question is the serum
complement. Several workers have shown that this is
not a simple substance, but one composed of at least
four components, two of which are associated with the
globulin fraction and two associated with the albumin
fraction. According to the present conception of the
action of complement, all components must be present
before it can cause the lysis of sensitized cells.
The work of Moore and others in showing that complement
is an inheritable factor in guinea pigs, found that the
complement-free guinea pigs under their observation,
lacked the third component.

The value of this type of study lies in determining how low, in the zoological scale, complement is present in the animal blood, so as to trace, if possible, the development of animal groups by means of this inherited factor. If this factor has arisen as a mutation at some point in animal development that point should be determined.

The reported evidence available at the present, has demonstrated the existence of this complementary substance in Mammals, Aves, Amphibia and the Pisces. It is well known that some normal animal serum of animals in phyla lower than the Chordates exhibits a lytic action on erythrocytes. It is yet to be definitely proven that a complement exists in these sera. This work should be extended to the simplest form of animal life, to find, as has been suggested, the point in the evolutionary scale where it first appears. It is true that the results might not be very enlightening but the effort would be worth while.

Another point of value which might come from an extended survey of the presence of this substance would be in determining the source of complement. By an anatomical comparison of all animals which possess complement in their blood an elimination procedure could be resorted to in order to determine whether or not certain organs or tissues might be responsible for the

production of this substance.

A point which should be mentioned relative to the demonstration of complement in animal serum is one raised by Marsh and others following experimental work on the effect of food on the complement titer of guinea pigs. Marsh found that when ascorbic acid is withdrawn from the diet that the complement disappeared or was greatly reduced in titer within seven days. This time period is too short to permit malnutrition to cause this effect. Upon restoration of vitamin C to the diet the complement titer rose to normal titer within a weeks time. From these results it is evident that one must consider several factors if complement cannot be demonstrated in animal serum. It is possible that under other conditions complement might be present, therefore negative results must be checked under a variety of conditions.

This work demonstrated the presence of complement in members of the Reptilia thus providing a link, in the survey of the distribution of complement, which had not been demonstrated. The possibilities that a complete survey offer, justifies the preceeding experimental work. It is hoped that additional work can be carried out at a later date showing additional distribution of complement in the Reptilia.

The third line of investigation which was suggested in the beginning of this discussion applies very materially

to the experimental work. That of the distribution of
normal hemagglutinins and normal hemagglutinogens in
animal sera. These substances also are specific inherit-
able factors, associated with the globulin fraction of
the serum and the cells of the circulating blood.

The question of normal antibodies is one which has
not been settled. Various types of normal antibodies do
exist in animal blood, and in considering this subject
as a whole, the following suggestions have been made in
regards to their origin. 1. They may appear following
an unrecognized infection. 2. They may result from the
ingestion and ultimate absorption of antigenic substances
by means of food. 3. The presence of Forssmans Antigen
in bacteria and in foods. 4. Normal antibodies arise by
spontaneous origin as evidenced by certain regularities
in their distribution and 5. Normal antibodies are form-
ed as physiological genetically determined substances.

A brief mention should be made of the relationship
of normal antibodies and serum proteins. In the chick
embryo it has been found that normal hemagglutinins
appear at about the 11-12 day. This compares favorably
to the appearance of the globulin fraction of the serum.
This supports the evidence of considering these as
physiological genetically determined substances. It
is suggested that the source of serum proteins and of
antibodies may be one and the same.

The last of the above suggestions is one which willl be considered primarily in connection with the experimental work, since we are dealing with normal hemagglutinins and normal hemolysins which have been definitely proven to be inherited in a true mendelian manner.

The question of the distribution of normal hemagglutinins and hemolysins has been studied by many individuals. The statistical data at the present time is not organized in a very definite manner, so as to give valid and valuable information. If such work could be concentrated along one special line, perhaps some definite information could be realized instead of a chaotic mass of meaningless data.

Since the isoagglutinogens and isoagglutinins in man have been proven to be specifically inherited factors, these should offer a definite line for investigating the presence or absence of similar factors in lower animals. With such a possibility in mind, the experimental work of parts II and III was carried out. It was hoped that any contribution made would increase our meager information concerning the distribution of these factors and help to provide a method of studying the serological relationship of man and lower animals.

The current conception for the presence of the agglutinogen factors A and B, is that they have arisen as mutations in man. This conception is based upon the fact that the most primitive races of man show a great

preponderance of type O individuals an those not possess-
ing agglutinogen factors, but possessing both agglutinat-
ing substances alpha and beta. A notable exception to
this is the tribe of the "Blackfeet " Indians. This
exception may be explained on the basis of race impurity,
since it it well known that this tribe of Indians are
notoriously an immoral group.

The evidence presented by a survey of the literature
concerning the distribution of these factors in animals
offer an interesting bit of comparison. Let us see what
factors have been demonstrated in lower animals by various
individuals. In man, of course we find four factors,
the two agglutinins alpha and beta, and the two agglutin-
ogens A and B. In anthropoid (61), (57) apes it has been
generally found that they fit into the same grouping as
man, thus having the same four factors. Alpha, beta, A
and B. In the lower monkeys the grouping is not distinct
and of the agglutins, only alpha (62), (63) has been
definitely demonstrated and of the agglutinogens, a B
factor, at least similar but perhaps not identical to
human B factor, has been demonstrated. In animal groups
lower than monkeys, we find very little information.
Lower (64) found that the agglutinins and agglutinogens
of dogs are similar to human group B and therefore would
contain alpha and B factors. In rabbits (69) group
specific agglutins for human A and B cells have been

demonstrated thus alpha and beta are present. Herman (20) found a resemblance between human and horse agglutinogens and Gorer (59) found a close relation between mouse agglutinogens and the A, and A_1 factors of man.

Below the group of the mammals information is extremely meager. Karshner (52) found that chicken sera constantly agglutinated human cells but that human sera seldom agglutinated chicken cells. Although no definite statement is made in regard to what factors are present, these results would indicate the presence of agglutinins similar to human alpha and beta, and the absence of agglutinogens. This is the only reported information available for the Aves.

This is as low in the zoological scale as the published literature covered and it is with animals below this group that this experimental work was done. The results obtained with the Reptilia compare very favorably to those mentioned above. This work demonstrated the presence of specific absorbably agglutinins in snake serum for human A and B cells, but the absence of agglutinogens in snake erythrocytes for human alpha and beta agglutinins.

From all of these results we must conclude that hemagglutinins similar to or identical with human alpha and beta hemagglutinins are found in at least the lower mammals, Aves and the Reptilia. The agglutinogen factors may or may not exist in lower mammals, but are absent in groups lower than mammals.

It must be understood that there has been such a
small amount of work done on the lower groups that the
results are only indicative. An inexhaustable field is
open to investigators to continue along this line of
work. The group of the Aves must be examined additionally
and more specifically than the work of Karshner. The
Reptilia presents in itself a field of such magnitude
that it is almost impossible for one person to corner
it. It is the hope of the writer that similar work, to
that carried on for the Serpentes, can be extended soon
to the other orders of this group of animals. Perhaps
the other types of Reptilia as the turtles, alligators
and lizards are serologically similar to the snakes.
Such an investigation is the next planned by the writer.
This still leaves the two lower groups of Chq dates, the
Amphibia and Pisces to be examined in a similar way.
So we must consider all of this as only a very small
beginning in a field which offers great possibilities for
the immunologist and the anthropologist. Eventually
we can hope for answers to questions such as the follow-
ing . What is the distribution of the isoagglutinating
and isoagglutinable factors of man, in the animal kingdom?
Are these isoagglutinins and isoagglutinogens entities
which have arisen by mutation in some stage of animal
development? Can we trace any more definitely the
lineage of man by this procedure than by our vast
knowledge of comparative anatomy and paleontology?

Can the Science of Serology be considered as a very
definite part of Biology? These questions are of prime
importance and must be kept well to the front by any
person continuing investigations along the above suggest-
ed lines of endeavor.

In addition to the definite results mentioned above,
I have discussed briefly in the section on experimental
procedures, some interesting incidental observations.
It is possible that these observations may later be
utilized in some way.

It was observed that centrifuged samples of snake
serum showed a much greater volume of serum than of
packed cells. The average for forty specimens was
found to be 74.26% serum and 25.74% cells. It is
suggested that a blood with such a high serum content
might be utilized in the study of the origin of blood
proteins. Also a comparative study of the serum proteins
of man and lower animals might prove to be of value.

A second interesting observation was made in regard
to the clotting time of snake blood. It was found that
the time is considerable greater than that of man and
common laboratory animals. It is possible that such
information could be utilized in the study of the
mechanism of clotting. In any exent a comparative study
of the two procedures would be extremely interesting.

I believe the preceeding experimental work is
justified because of the following contributions to
the study of the serological relationship of animals.

1. A missing link in the chain of evidence concerning
   the distribution of complement in animals has been
   supplied. That for the group of animals the Reptilia.

2. The information concerning the distribution of
   hemagglutinins and hemagglutinogens, comparable to
   those of man, has been advanced one more step in
   that hemagglutinins similar to the alpha and beta
   factors in man were demonstrated in snake serum,
   and that snake erythrocytes do not contain agglutinogen
   factors similar to the A and B factors of man.

Since both of these reasons can be utilized in the
study of the evolutionary development of man the writer
feels that these contributions are of some value to
the sciences of Immunology, Genetics and Anthropology.

In addition to extending our present information,
the preceeding work has suggested several problems
which the writer hopes to carry out in the future.
This field of endeavor seems to be an almost inexhaust-
able one, so will necessitate the limiting of the
more immediate work to the chosen phylum of animals,
the Reptilia. The following problems present themselves
here.

1. Is complement a constituent of the blood of other
   groups of Reptiles as the turtles, lizards and

alligators?

2. Are the hemagglutinins for human cells also found in other types of Reptiles?

3. Are the hemagglutinogens of human cells present in the erythrocytes of other Reptiles?

4. By means of the precipitin reaction, what is the serological relationship of the various species of snakes and their serological relationship to other animals?

5. Is the high anticomplementary titer of normal human serum and the low anticomplementary titer of syphilitic human serum for snake complement of any diagnostic significance?

6. Why is it impossible to reactivate heated snake serum with guinea pig complement?

Additional problems which the writer may eventually be able to consider are.

1. The distribution of complement in animals below the Chordates.

2. A study of hemagglutinating substance for human cells in the Amphibia and Pisces. Such may be extended to other phyla.

3. A suggested problem from the discussion of experimental data is one of a study of the delayed clotting time of snake blood.

4. Another more unrelated problem is one of a study of the types of curculating blood cells in the Reptiles.

Conclusions

The preceding experimental work justifies the following conclusions.

1. Isohemagglutination does not occur in snakes.

2. Heterohemagglutination between families of the Serpentes does occur.

3. Snake cells do not contain agglutinogens which will be agglutinated by human alpha and beta agglutinins.

4. Snake serum may or may not contain agglutinins for human erythrocytes.

5. Agglutinins are found in some snake serum which are specific for human A and B agglutinogens.

6. Certain snake serum contains a species agglutinin for human erythrocytes.

7. Appropriately absorbed snake serum may be used to determine human blood groups.

8. Snake and human hemagglutinins are equally thermostabile.

9. All fresh snake sera examined exhibited a lytic effect on human and sheep erythrocytes.

10. Snake serum contains a thermolabile complement and a thermostabile hemolysin.

11. Snake complement compares very favorably to guinea pig complement in its activity, titer, and stability.

12. This complement from cold-blooded animals
    will act on cells sensitized with hemolysin
    prepared in a warm-blooded animal.

13. Heated snake serum can be reactivated by fresh
    snake complement but not by guinea pig com-
    plement.

14. Normal human serum is highly anticomplementary
    for snake complement, but human syphilitic
    serum is only slightly so.

15. The hemolytic titer of fresh snake serum depends
    upon the titer of the sensitizer rather than the
    titer of the complement, since the complement is
    active to a higher dilution than the sensitizer.

16. The average percentage for the constituents of
    40 samples of snake blood was found to be, serum
    73.93% and cells 26.07%.

17. Snake cells show a much more rapid sedimentation
    rate than human cells.

18. Snake blood clots much more slowly than the
    blood of man and the common laboratory animals.

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