FIELD & LABORATORY

Volume XXIII April 1955 Number 2

The Use of Saline-soluble Tissue-proteins in Systematic Serology

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In recent years the emphasis of taxonomic studies in many animal groups has been at or near the species level, with often little done to clarify the relationships of the higher taxonomic units. This was either because it was felt that these units needed no revision, or that there was not enough new information at hand to warrant any revision. In many cases, groups of genera have been placed in a family simply because of some superficial resemblance.

Authorities in these groups have neither denied nor overlooked the possibilities of convergence and parallelism in such instances. They have had to base their theories of relationship on such evidence as was available to them; and detailed studies of anatomy, physiology, and life history were often lacking.

Illustrations of the above may be found in several fields. Within the avian order Passeriformes, for example, the relationships of many groups of birds are poorly understood, and most competent students are agreed that some of the families of current classifications are artificial groups. They came from earlier work that emphasized external structures as taxonomic characters, like size and shape of the bill, now recognized as highly adaptive structures which frequently show convergence and parallelism.

As such adaptive features often fail to give a clear understanding of animal relationships, one is forced to consider other features—morphological, physiological, and ontogenetic—in any attempt to ascertain true relationships at higher taxonomic levels.

Since all features, morphological, physiological, and others, are subject to forces of the environment, convergence and parallelism may never be ruled out completely. Such features as are more conservative and change more slowly in response

to environmental forces are the ones which, if found, can be of value in determining relationship.

As chemical systems within the body of an organism are relatively stable, the amount of change which can occur in such a system is relatively slight. Such things as pH, ionic balance, and types of protein compounds remain constant; and within a species proteins are alike. Between species, there are differences in proteins; but enough work has been done to indicate that the more closely related animals are, the more similar are their proteins. This fact has formed, in part, the basis of systematic or comparative serology.

Application of serological techniques to the problems of animal relationships has been attempted with varying degrees of success. While but few of the earlier studies were quantitative, serological techniques of a quantitative nature have been developed within the past decade by which taxonomic relationships may be estimated. Their usefulness has been demonstrated in many groups where results obtained comported with results obtained by other methods, such as comparative morphology. Boyden (1942) in an excellent review of the use of these techniques has stated that "comparative serology . . . is no simple guide to animal relationship." Nevertheless, the objectivity of method, the fact that it has its basis in the comparisons of chemical systems that seem to change but slowly in response to environmental pressures, and the further fact that the results are quantitative, favor its use as a means to determine group-relationships of animals, particularly when other evidence is lacking or of uncertain value.

Serological techniques in vertebrate systematics have not been extensively employed. Mammals have received the most attention. The precipitin technique is the one used most extensively; and blood proteins are the ones that have been investigated most thoroughly. The use of blood proteins, however, had limited serological methods to the study of those animals with large quantities of blood. As this involved large animals, or animals which could be collected in large numbers, the use of *tissue* proteins became inevitable, and they have been used with some success by several workers. Martin & Leone (1952) worked with birds of known relationship and showed that the "accepted" systematic positions of these animals were confirmed by serological procedures involving

tissue proteins. Stallcup (1954) applied these techniques to an actual problem in avian taxonomy, and the data obtained by the use of these methods were of great value in his attempt to clarify the relationships of the species involved.

Since the application of serological techniques employing tissue rather than blood proteins to the study of animal relationships has proved successful, many other groups of animals may now be studied by this method, where quantities of blood are not available. For this reason the techniques used in such studies are described below. It must be pointed out that these techniques need some revision. Careful study is now being made of the different phases, in an attempt to improve the methods and thereby increase the value of the results.

When an animal is collected, the entire digestive tract should be carefully removed to prevent the escape of digestive enzymes into the tissues and to prevent putrefaction by action of intestinal bacteria. If the specimen is not to be used immediately, it should be wrapped carefully in aluminum foil (or treated otherwise, to prevent dehydration of tissues) and frozen quickly. It should be kept frozen until the time when the extract is to be made. When an antigen is to be prepared, the specimen should be allowed to thaw but not to become warm. The tissues may then be minced in cold 0.9 per cent aqueous solution of NaCl. A Waring blender is effective in reducing the tissues to small pieces. The amount of saline used for extraction will vary from one type of tissue to another, but Stallcup (1954) used 75 ml. of saline for each gram of tissue to be extracted. Extraction of the proteins should be allowed to take place over a period of from 48 to 72 hours at low temperature (2°C.). At the end of this period the tissue residues can be removed by centrifugation. The supernatant must be clarified and sterilized. Use of a Seitz filter will accomplish this. The filtrate may then be bottled and should be stored at a temperature of 2°C.

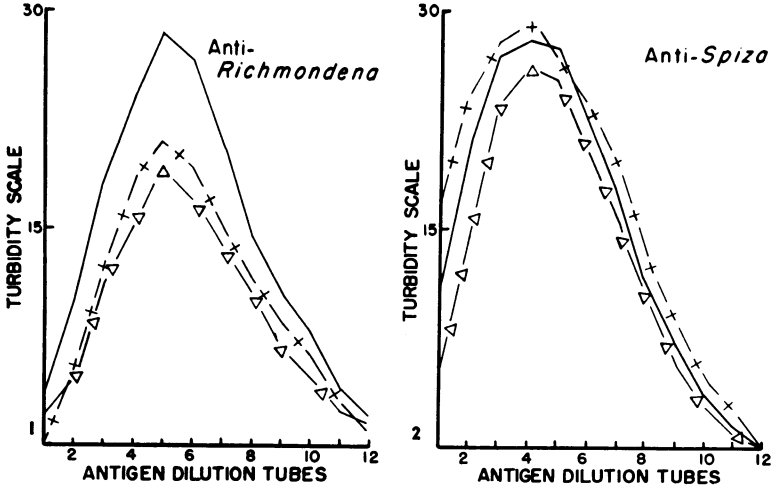
Antisera are produced in rabbits. Differential effects of intravenous, subcutaneous, or intraperitoneal injections are not clear as yet. All three types cause antibody production. Each rabbit should receive one or more series of four injections, each injection being administered on alternate days and doubling in amount: 0.5 ml., 1.0 ml., 2.0 ml., and 4.0 ml. On the eighth day after the last injection, 10 ml. of blood

are withdrawn from the main artery of the ear of the rabbit, and the antiserum may be used in a homologous precipitin test (see below) to determine its effectiveness. If sufficient amounts of antibodies are not present, another series of injections can be given. When the antiserum contains sufficient amounts of antibodies for the projected tests, the rabbit must be bled to death by cardiac puncture. The whole blood should be placed in clean test tubes and allowed to clot. After the serum has been expressed, it may be decanted and centrifuged to remove all blood cells. It must then be sterilized by filtration through a Seitz filter, placed in sterile vials, and stored at 2° C. until used.

The precipitin reaction occurs because antigenic substances introduced into the body of an animal cause the formation of antibodies which precipitate the antigens when the two are mixed. The antisera produced show quantitative specificities in their actions; therefore, when an antiserum containing precipitins is mixed with each of several antigens, the reaction involving the homologous antigen (that used in the production of the antiserum) is greater than those reactions involving the heterologous antigens (antigens other than those used in the production of the antiserum). Moreover, the magnitude of the reactions between the antiserum and the heterologous antigens varies according to the degrees of similarity of those antigens to the homologous one.

When a series of precipitin tests is to be carried out, the amount of protein in each antigen should be measured. This may be done colorimetrically by one of several tests involving the use of a clinical colorimeter (Leitz Photrometer, Bausch & Lomb Spectronic 20). Each test should consist of at least 12 test tubes, the size of the tube depending on the amount of sample to be used (which, in turn, depends on the instrument to be used in measuring the turbidities developed—see below.) The first tube should contain an initial dilution of the antigen of arbitrary protein concentration (for example, 1 part protein in 250 parts saline), and each successive tube should contain a protein dilution one-half the concentration of the preceding tube. The antigens of all such tests should be of the same concentration. Antigens may be diluted and concentrations adjusted with 0.9 per cent saline solution. To each tube is added the same amount of anti-

serum. Saline controls, antigen controls, and antiserum controls should be maintained with each test to determine the turbidities inherent in these solutions. Turbidities developed as a result of precipitin reactions are allowed to develop over a period of 24 hours. At the end of this period turbidities may be measured by any refined turbidimeter (Libby Photron-reflectometer, Spectronic 20, Nephalometer.) If no steps are taken to prevent bacterial growth in the tests, the precipitin reaction should be allowed to take place at a low temperature (2° C.) Bacterial growth may be prevented by the addition

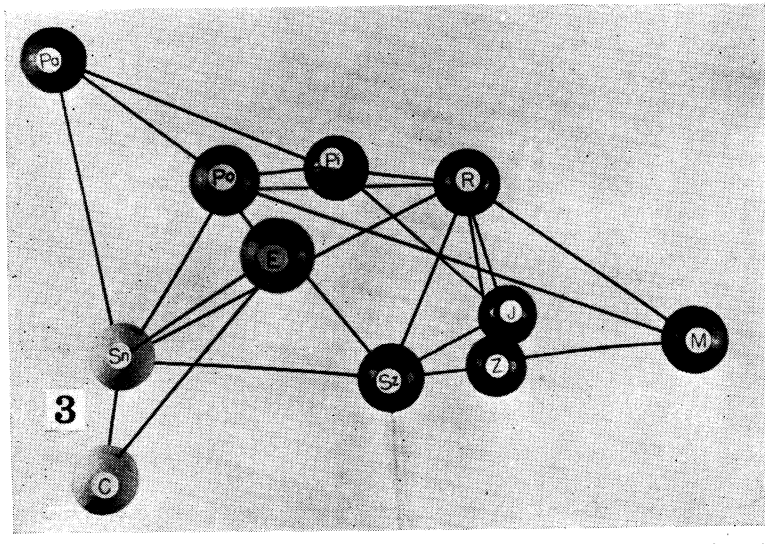


to each tube of "Merthiolate" (Thimerosal, Lilly) in a final dilution of 1:10,000. This has an advantage in that the tests may be run at room temperature, and greater turbidities may develop.

Corrected values for the turbidities obtained may be plotted with turbidity values on one axis and antigen dilutions on the other. The homologous reaction is the standard of reference for all other test reactions with the same antiserum. By summing the plotted turbidity readings numerical values are obtained which serve as indices characterizing the curves. Such values may be converted to percentage values, that of the homologous reaction being considered 100 per cent. These values, plus the curves, provide the data by means of which the proteins of the animals tested may be compared. Because different rabbits produce different anti-

sera against the same antigen and under the same conditions, actual comparisons may be made only among values obtained from tests made with the same antiserum.

In analyzing the serological relationships of the species used in a study of this type, it should be emphasized that two or more series of tests must be considered before the animals can be placed in relation to each other. To illustrate this point by a hypothetical example, two species might seem equidistant serologically from a third species. Additional testing should indicate if the first two species are equidistant in the



same direction (therefore, by implication, close relatives) or in opposite directions (therefore, distant relatives.) A single test supplies only two dimensions of a three dimensional arrangement. An actual example may serve to emphasize further this point. The data presented in Fig. 1 indicate that *Spiza americana* (dickcissel) and *Molothrus ater* (cowbird) show approximately the same degree of serological correspondence to *Richmondena cardinalis* (cardinal). This does not imply necessarily that *Spiza* and *Molothrus* are closely related. If Fig. 2 is examined, it can be determined that *Richmondena* shows much greater serological correspondence to *Spiza* than does *Molothrus*. By reference to other series of tests involving these three species a more exact determination of their relationship may be obtained.

It is impossible to interpret and picture data of this sort satisfactorily in two dimensions. Fortunately, the data lend themselves well to a three dimensional interpretation. Since the data are quantitative, it is possible to construct three-dimensional models (Fig. 3). By use of the percentage values obtained by the precipitin tests, each animal may be located on the model in relation to the others. Such a model serves to summarize the relationships of the species tested.

In summary, the use of serological techniques in the study of saline soluble proteins extracted from the tissues of animals has been found to be of value in the determination of animal relationships. It is not suggested that such methods of study be used to the exclusion of others, but that these methods may yield important data which, when used along with data from other fields, may help to give a clearer idea of the relationships of the groups being studied.

LITERATURE CITED

BOYDEN, A. (1942). Systematic serology: a critical appreciation. *Physiol. Zool.* 15:109-145

MARTIN, E. P. & LEONE, C. A. (1952). Serological relationships among domestic fowl as shown by comparisons of protein preparations from corresponding organ systems. *Trans. Kansas Acad. Sci.* 55:439-444.

STALLCUP, W. B. (1954). Myology and serology of the avian Family Fringillidae, a taxonomic study. Univ. Kansas, *Publ. Mus. Nat. Hist.* 8:157-211.

Bee Visitors of Penstemon ellipticus (Scrophulariaceae) on Eddy Peak, Sanders County, Mont., in Summer of 1954

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Eddy Peak, in the Cabinet National Forest of northwest Montana, is part of a large fault system in which thousands of feet of shale were thrust up to form an escarpment overlooking the Clark Fork River. The summit elevation is 7500 feet. Fracturing of the uppermost layers of shale has resulted in many talus slopes, some of them hundreds of yards from top to bottom, on the dip side of the strata. Parts of the mountain crest consist of nothing but a jumble of shale blocks in a very unstable state. During the summer of 1954 several slides occurred. Apparently for this reason, vegetation was sparse on the slopes, and heavier on the cliff side. The only plant which could be found on both sides of the crest was *Penstemon ellipticus* Coult. & Fisher. For the first two weeks of July the mountain top was almost wholly covered by snow, and snow persisted in shaded areas throughout the