SOME MOLECULAR APPROACHES TO TAXONOMY

DR. WAYNE FRAIR

The King's College, Briarcliff, N. Y., 10510

A method for studying the proteins of various turtles is given, based essentially on production of antiserums by injection of turtle blood into rabbits or chickens. When mixed with serial dilutions of serum from various species of turtles, varying degrees of turbidity or precipitation are obtained. These results are shown to have a definite taxonomic value and do not support the present widely-held position that snapping turtles belong to a separate family related to the Kinosternidae, but rather are in the Emydid family group.

A method of comparing DNA of various species by studying the amount of pairing of DNA strands in agar in relation to a standard "reference" DNA is described. Since DNA consists of an "alphabet" of only four letters, until we can learn to read the "words" made by sequences of any three of them, it would seem that more progress in unraveling molecular taxonomy can be made by studying proteins, built from an "alphabet" of twenty letters or amino-acids. Rather than use evolutionary presuppositions, this research proceeds from the working assumption that the world of life is to be viewed as having arisen from certain stem organisms or "kinds" which in most cases need to be elucidated.

Introduction

In order to detect the pattern of living things in nature, the earliest classifiers used the unaided eye in their study of *macroscopic* anatomy. The advent of magnifying lenses about four centuries ago made it possible to study *microscopic* anatomy (which included details of developmental anatomy or embryology). Most recent approaches, which had their beginnings at the turn of the 20th century, seek to determine relationships among living things using a molecular approach–what might be called "*molecular* anatomy."

Usually molecules can not be observed (even with an electron microscope) and so it has been necessary to utilize antibodies or various types of instruments to recognize similarities and differences among these molecules.

Molecular substances most commonly used have been the large or macromolecules, namely proteins, and then very recently DNA. Each type of organism has specific kinds of proteins and DNA, but proteins and DNA are found to differ when one group of living things is compared with another group.

Most of the available information on taxonomic biochemistry and serology, with exception of the latest DNA experiments, is summarized or at least bibliographically referred to in a symposium volume[7].

In living things, DNA determines the "anatomy" of proteins, which in turn play roles of primary importance as structural and functional (including enzymatic) proteins in a multitude of chemical-physical conditions basic to the development, anatomy, physiology, and behavior of the organism. Therefore, when it is possible to characterize the proteins and DNA—for these chemical substances basically are responsible for life as we know it—we should have a clear picture of the creative pattern in nature. An outcome of studies on macromolecules should be the characterization of nature and detection of homologies in Owen's sense of "essential similarity"[1].

In initiating a program pointing in this direction, I began working with the reptiles, for they are considered by evolutionists to occupy an important position between amphibians, on the one hand, and birds and mammals, on the other. The reptilian order, Chelonia, which contains all turtles has been a neglected group; and, therefore, since it has been possible to obtain many types of turtles, I increasingly have utilized them in an expanding program aimed at clarifying their molecular taxonomy.

Proteins in Turtles

Blood has been collected by cardiac puncture without serious trauma, and serum obtained from the blood has been injected into rabbits or chickens in order to produce antiserum against the turtle serum proteins. Antiserum was mixed with serial dilutions of various turtle serums; mixtures were incubated for 20 minutes at 37° C and then checked quantitatively for precipitation (turbidity) using light scattering (Leone, p. 537).

In preparation for the test for which results are shown plotted in Figure 1, *Chelydra s. serpentina* (common snapping turtle), serum was injected into rabbit #32; after several weeks antiserum for *Chelydra* was obtained from the rabbit and was mixed with doubling dilutions of serum from snapping turtle (reference antigenic material). The same antiserum also was mixed with serum from the following animals (cross-reacting antigenic material) whose scientific names are shown below the common snapping turtle (from top to bottom) on the

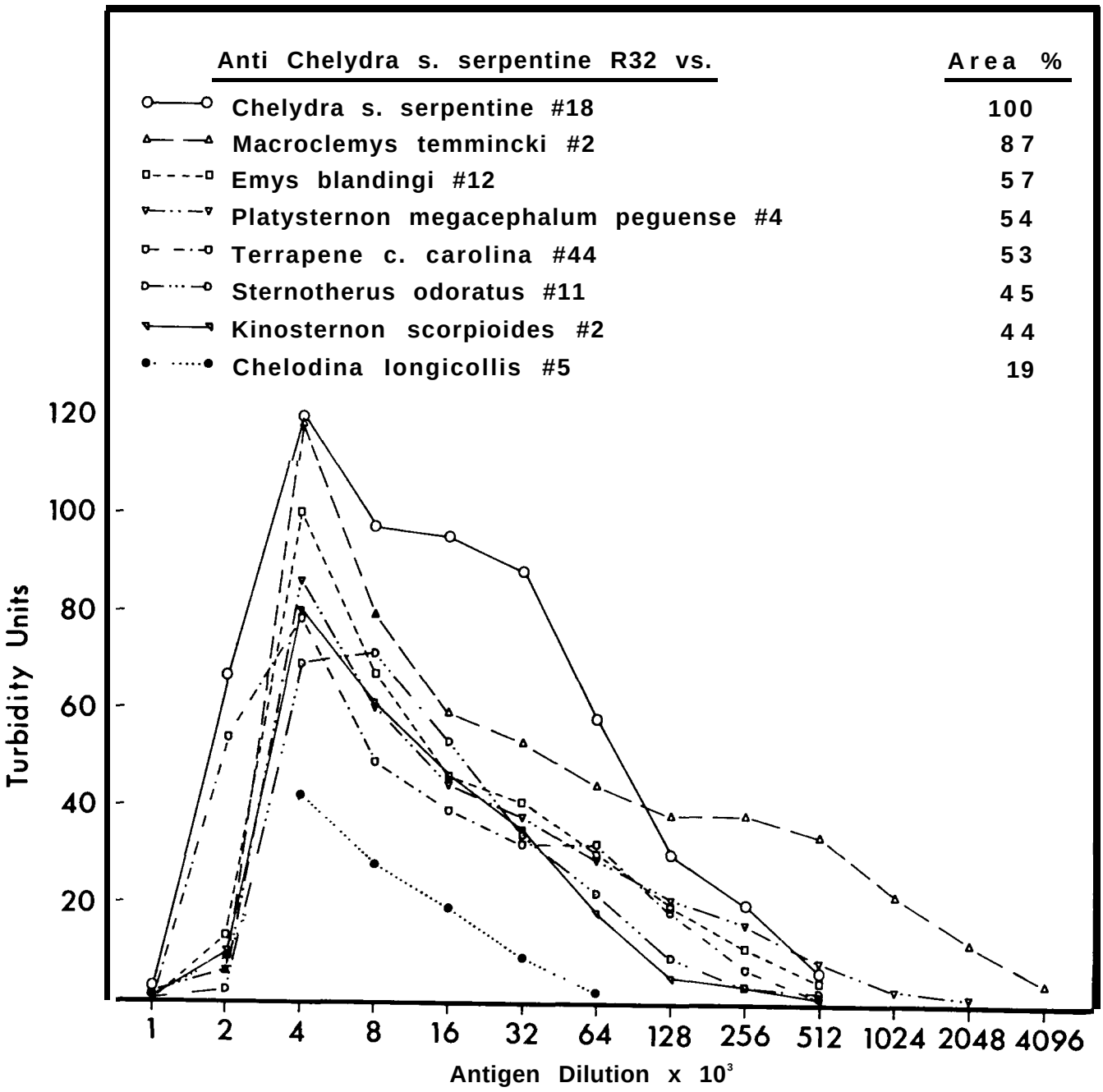


Figure 1. Plots of turbidity values over antigen reaction ranges after rabbit anti-snapping turtle serum was mixed with doubling dilutions of serum from each of 8 turtles.

graph: alligator snapping, blandings, big-headed, eastern box, musk (stink-pot), mud, and snake-necked turtles.

Relative areas under the graphs are expressed in the Area % column using the *Chelydra* area as 100%. These values express the order of relationships existing among serum proteins in these animals and when enough of these data are accumulated they help in determination of the "kind" of turtle(s) ancestral to others.

Presently these results have a definite taxonomic value. They do not support a current widely-held position (q.v. e.g. Romer) that snapping turtles belong in a separate family which is related to the Kinosternidae family containing mud *(K. scorpioides, 44%)* and musk (S. *odorattm*, 45%) turtles. These results clearly show the snapper to be closer to the emydid

family which contains the blandings *(E. blandingi,* 57%) and box *(T. c. carolina,* 53%) turtles.

The great advantage of these precipitation evaluations is that with a series of readings obtained from the results of two reacting solutions (one being serially diluted), an investigator is measuring the sum of many antigen-antibody (an-ab) reactions. When the an-ab fits are good, there is considerable precipitation, and when the an-ab matching is not as good there is less precipitation.

Apparently the method quantitates degrees of similarity of a whole spectrum of proteins; and this method, therefore, obviates problems of relationship which can arise when single proteins are compared either immunologically, or after study by certain analytical methods. Essentially the technic simultaneously is discriminating among "anatomies" of one or two dozen or more specific proteins. The systems which are acting actually can be determined by other methods including immunoelectrophoresis.

However, there are disadvantages. Without detailed analyses, we are not sure which an-ab systems are reacting in each case. What we have been calling quantitative comparisons are only *relative* values; for with other dilutions of the same antiserum, or with different antiserums, other percentages will be obtained. In spite of these disadvantages, the values obtained usually are repeatable within 5% and show the same relative order when different antiserums are utilized.

In addition to many other studies like the above, which utilizes precipitation in fluid systems, my results using solid media such as agar have agreed qualitatively with quantitative values as shown in Figure 1. Electrophoresis studies also have given helpful qualitative results[9]. In this type of study, the leading anodal component of reptiles appears to be more important as a taxonomic indicator than the proteins of larger size which group toward the cathode. In paper and cellulose acetate (barbital buffer, pH 8.6), kinosternids show fast-moving anodal components similar to human albumin whereas the leading component of serum from chelydrids and emydids is farther back from the anode. So here again, snappers are more like emydids.

In Figure 2 the five animals (top to bottom) are eastern box, common snapping, alligator snapping, eastern mud, and musk turtles. The leading anodal components (albumin) of the snapping turtles are seen to be positioned more like that of the box turtle than like mud and musk turtles. For a reference standard, the human pattern is shown; it is **not** for indicating relationships between man and turtles.

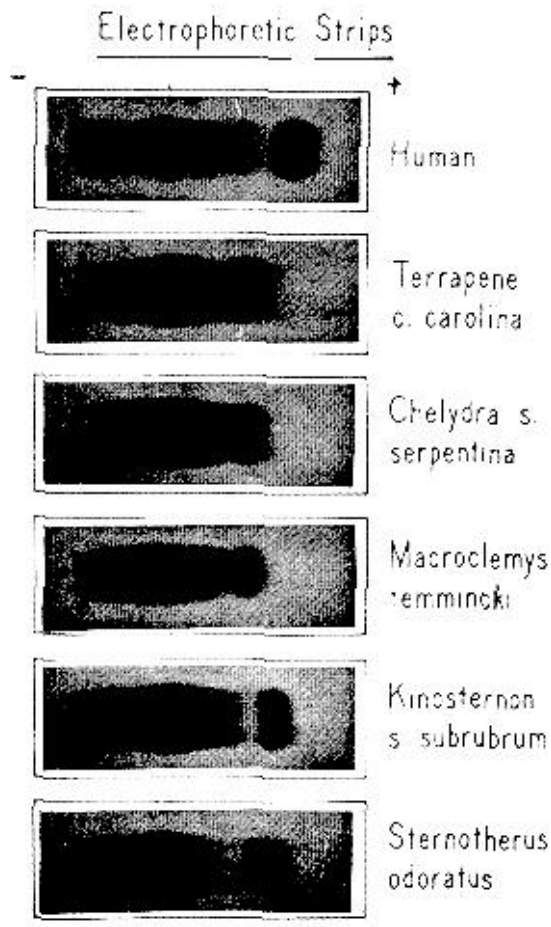


Figure 2. Results of electrophoresis of pooled turtle serums using cellulose acetate membranes. Serum was applied where marked just left of center; anode (+) at right; cathode (—) at left.

DNA—Use in Taxonomy

Because differing protein structures are determined by the base sequence in DNA, it has been felt that if we could read the DNA of each organism we would have a most basic approach to the study of organisms. DNA, believed to be responsible for the hereditary characteristics of living things is remarkably stable, and now it appears that cells even have the ability to repair damage caused to DNA by mutagenic agents.[3]

Considerable research is directed toward understanding DNA, but so far it has not been possible to read DNA "blueprints." However, within the past five years a technic has been utilized for obtaining relationships among living things based upon the degree of matching of separated strands of DNA from different organisms[2,4,5,6,8]. The technique involves the following steps:

1. High molecular weight DNA is heated to separate the two strands and then it is cooled quickly to prevent rejoining.

2. The single strands are embedded in agar which is then sieved, and the granules are divided into several equal portions.

3. Other samples of DNA from the "reference" material (DNA used in steps 1 and 2) and from what will be "cross reacting" material are sheared to much lower molecular weight.

4. The sheared material is heated and cooled to get single strands.

5. Quantities of sheared, single-stranded DNA from several origins then are incubated each with a portion of the agar particles containing DNA from the reference' material. The small filaments diffuse into the agar and join with complementary regions of the trapped longer filaments.

6. The agar is washed to remove small strands which are not paired with the longer trapped filaments.

7. Then the agar particles in the several groups are evaluated to determine the quantity of the several types of specific small strands which have duplexed with the large trapped strands from the reference material (Figure 3).

The method has been used in studies involving annealing not only of DNA-DNA but also of RNA-DNA. RNA has been found to be complementary to only one of the DNA strands, and DNA has been shown to possess regions complementary for transfer, messenger and ribosomal RNA.

Studies have been done of annealing reactions among various types of organisms as well as with different DNA species from the same cells. It appears that different DNA molecules exist within a single cell, as *Euglena gracilis*, where nuclear, chloroplast, and mitochondrial DNA may be distinguished by size and base composition. Some of the procedures used in this research have involved certain modifications of the aforementioned technic, for instance utilizing nitrocellulose membranes instead of agar[10].

Cross-reacting values are compared with the value obtained in the reference reaction, and results are believed to indicate quantitative degrees of resemblance among the types of low molecular weight strands annealing with the high molecular weight reference material. Microorganisms have been utilized in most of the studies so far, but certain investigators have included even a variety of vertebrates as shown in Table 1. As would be expected from gross anatomical examination, those animals showing more overall structural similarities such as mouse

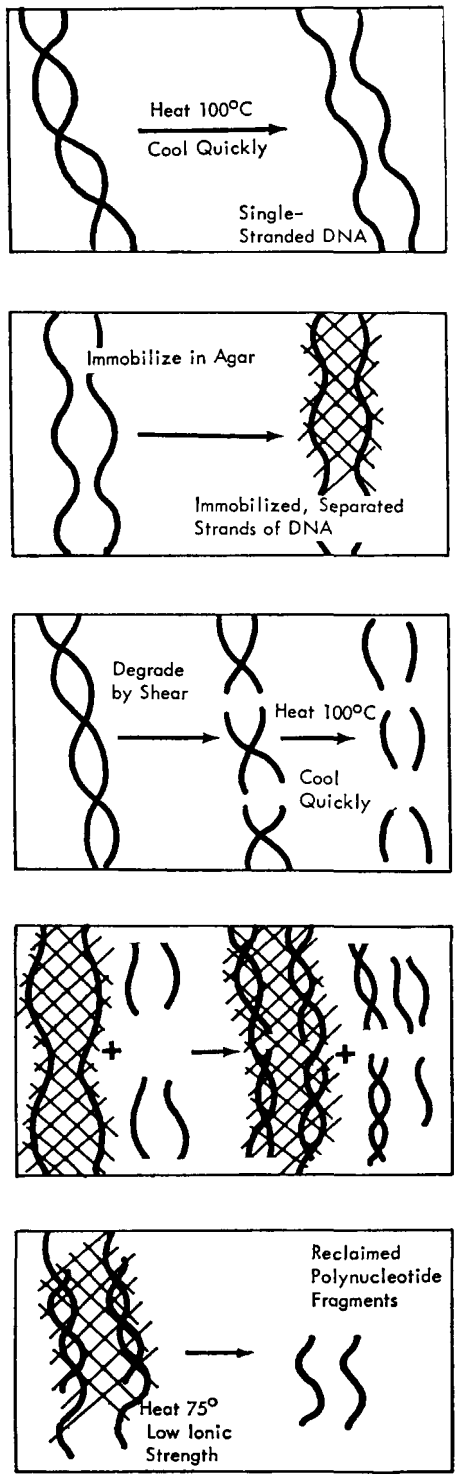


Figure 3. Diagrammatic representation of the principle of the DNA-agar procedure. (Reprinted by permission of *Science* and of Hoyer[6], p. 960.)

and rat showed higher percentages of duplexing of their respective DNA's.

Table 1. Percentages of human and mouse sheared single-stranded DNA (fragments) bound in agar to high molecular weight single-stranded DNA from various organisms. (From Hoyers[5]).

DNA Embedded in Agar	Percent of Fragments Bound	
	Human	Mouse
Human	18	5
Mouse	6	22
Rhesus monkey	14	8
Rat	3	14
Hamster	3	12
Guinea pig	3	3
Rabbit	3	3
Bovine	5	4
Salmon	1.5	1.5
Escherichia coli	0.4	0.4
None	0.4	0.4

In our laboratory, Mr. John Cruzan conducted a study utilizing DNA which he had extracted from erythrocytes of some of the turtles shown in Figure 1 and certain others as well. So far, only emydid, kinosternid and chelydrid turtles have been used, but resulting hybridization values with the DNA have not been repeatedly different enough to permit differentiation among these groups. If more sensitive DNA methods can be applied, it may be possible to discriminate among these turtles even as has been done using serum proteins.

Discussion

Most comparative studies proceed with the assumption that similarity indicates descent from certain common ancestors. This approach is very questionable in many cases as for instance at the molecular level when we are dealing with specific individual proteins (e.g. insulin and hemoglobin).

Therefore, *many* types of macromolecules should be included in the best type of comparative study seeking to determine relationships among organisms. At present the detailed structure of too few proteins is known for this to be possible[6], and laborious procedures have been necessary for determination of amino acid sequences and other structural features. But progress is being made.

When techniques are perfected for conveniently sequentially cleaving off the terminal amino acids in polypeptide chains of various proteins, we will be able more readily to learn their structure. We will be able to compare the different proteins among living things, and be able to construct dendrograms showing the probable diversifications that have occurred since the beginnings of the various groups..

It seems to me that proteins offer us more hope of achieving a sound categorizing of nature than does DNA, at least at the present time. In the case of DNA we are dealing with polynucleotide chains built from an alphabet of only 4 letters. If we were able to read the DNA "words", presumably sound comparisons would be possible.

Since this can not be done yet, it may be better for molecular taxonomists to concentrate on the proteins, for in proteins we process polypeptide chains built from an alphabet of about 20 letters. With the larger protein alphabet, we actually have a magnification of differences existing in DNA strands; and, even for this reason alone, protein studies may remain as our most fruitful molecular approach.

It is obvious that nature contains recognizable groups of extinct and extant organisms, and for this reason classification is possible. That categories exist in nature is obvious, but that they share proximate or distant common ancestors is far less obvious in most cases.

Rather than using evolutionary presuppositions in our research, we are proceeding with the working assumption that the world of life is to be viewed as having arisen from certain stem organisms which in most cases need to be elucidated. We do not genetically cross gaps unless evidence is compelling. This impresses me as a judicious way to approach nature.

It is unnecessary and probably unwise to maintain an evolutionary tension, which often is expressed by a compulsion to bridge taxonomic gaps while describing nature. This may result from conscious or unconscious antitheological feelings or from a desire to conform to what is believed to be well-established procedures.

Whatever the case, all scientists must be prepared to re-evaluate the hypothesis (or theory) which they are using in integrating data concerning relationships of organisms. In determining taxonomic groupings we need all types of data—physiological, behavioral, developmental, and anatomical (macroscopic, microscopic and molecular). Molecular data are more prominent than ever before.

References

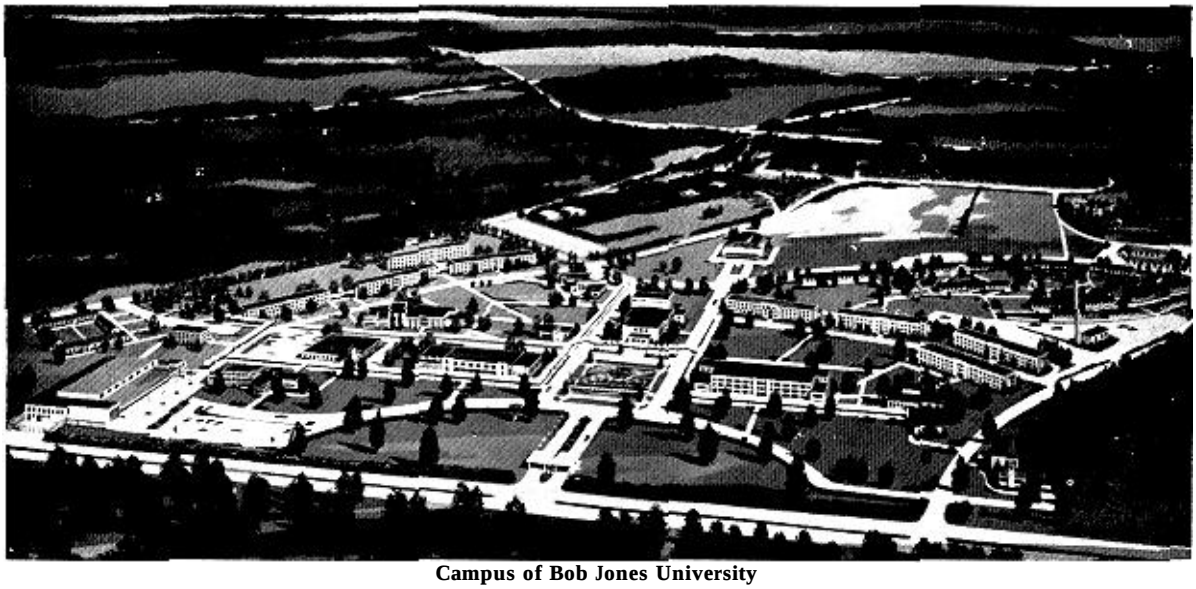
[1]Boyden, A., "Homology and Analogy. A Critical Review of the Meanings and Implicatlons of These Concepts in Biology," *American Midland Naturalist,* 37:648-669, 1947.

[2]De Ley, J , and I. W. Park, "Disslmilarity Between Human and Bacterial Deoxyribonucleic Acids," *Nature,* 211 :1002, 1966.

[3]Hanawalt, P. C., and R. H Haynes, "The Repair of DNA," *Scientific American,* 216:36-43, 1967.

[4]Hoyer, B. H., E. T. Bolton, B. J. McCarthy, and R. B. Roberts, "The Evolution of Polynucleotides," p. 581-590. In V. Bryson and H. J. Vogel, [ed.], *Evolving Genes and Proteins* Academic Press, New York, 1965.

(Continued on page 47)



Campus of Bob Jones University

creative activity by God, rather than the result of the action of random chance through vistas of time.

We are very pleased with such a forthright and clear-cut statement, and are happy to recommend this fine university to our members.

Bob Jones University is in Greenville, South Carolina. The institution grants the doctors degree for advanced studies in various departments, details of which may be obtained from Mr. Christ. Tuition is only $250.00 per year for 16 hours or less. Room and board per semester is $409.50. There is also a matriculation fee per semester of $40.00. Various laboratory, business machine use, and other fees range from $5.00 to $20.00 for organ practice. These are certainly very modest fees. As may be seen from the University layout, which unfortunately we cannot show in color, the grounds are beautifully planned, and evidently set in a wooded area.

It is a joy to know that such a fine Christian motivated College and University exists and we hope in future issues to feature others so that a wide range of institutions in various parts of the country may be presented.

(Continued from page 22)

[5]Hoyer, B. H., B. J McCarthy, and E T. Bolton, "A Molecular Approach in the Systematics of Higher Organisms," *Science,* 144 (3621) 959-967, 1964

[6]Jukes, T. H., *Molecules and Evolution*. Columbia Univ. Press, New York. 285 p , 1966. See also R. V. Eck and M. O. Dayhoff, *Atlas of Protein Sequence and Structure 1966.* National Biochemical Research Foundation, Silver Spring, Maryland. 215 p., 1966.

[7]Leone, C A, [ed.] *Taxonomic Biochemistry and Serology*. Ronald Press, New York. 728 p., 1964.

[8]McLaren, A., and P. M. B. Walker, "Discriminating" Power of Rodent Deoxyribonucleic Acid on Incubation in Agar." *Nature,* 211:486-490, 1966.

[9]Sibley, C. G., "Molecular Systematics: New Techniques Applied to Old Problems," *L'Oiseau et R.F.O.,* 35:112-124, 1965.

[10]Richards, O. C., "Hybridization of *Euglena gracilis* Chloroplast and Nuclear DNA," *Proceedings National Academy of Sciences of U. S. A.,* 57:156-163, 1967.