11.

The Relative Position of the Cetacea Among the Orders of Mammalia as Indicated by Precipitin Tests.

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(Text-figure 1).

There are still many unsolved problems in regard to the proper systematic positions of species, genera, families and orders of mammals. A reading of the frank and critical statements made by G. G. Simpson (1945) in his excellent report, "The principles of classification and a classification of mammals," will indicate the nature of some of the difficulties and the extent and diversity of these problems. With reference to the Cetacea, Simpson wrote as follows: (*loc. cit.*, p. 213-214):

"Because of their perfected adaptation to a completely aquatic life, with all its attendant conditions of respiration, circulation, dentition, locomotion, etc., the cetaceans are on the whole the most peculiar and aberrant of mammals. Their place in the sequence of cohorts and orders is open to question and is indeed quite impossible to determine in any purely objective way. There is no proper place for them in a *scala naturae* or in the necessarily one-dimensional sequence of a written classification. Because of their strong specialization, they might be placed at the end, but this would remove them far from any possible ancestral or related forms and might be taken to imply that they are the culmination of the Mammalia or the highest mammals instead of merely being the most atypical. A position at the beginning of the eutherian series would be even more misleading. They are, therefore, inserted into this series in a more or less parenthetical sense. They may be imagined as extending into a different dimension from any of the surrounding orders and cohorts."

It was this statement which stimulated us to undertake a careful comparison of the reactions of whale sera with those of other mammals, using a modern quantitative precipitin technique. Our hope was that evidence bearing upon the relative position of the Cetacea to other orders of mammals might thus be obtained, which, under the circumstances, could be of decisive importance.

MATERIALS AND METHODS.

ANTIGENS.

Sera or protein fractions of sera representing 17[1] of the 18 existing orders of mammals listed by Simpson (1945) have been tested. These sera have come from many parts of the world and have been obtained through the aid of many individuals and institutions. A list of contributing agents and agencies may be found under "Acknowledgments."

These sera have had various treatments and histories. The following kinds of materials were used for the production of suitable antisera, or for testing, or for both purposes.

1. Native fluid sera, Seitz filtered and stored in refrigerator.
2. Native sera kept frozen and then melted, Seitz filtered and stored in refrigerator.
3. Fluid sera preserved with formalin at a final concentration of 0.2% formalin.
4. Fluid sera preserved with merthiolate at a final concentration of 1:10,000.
5. Plasma with sodium citrate or oxalate, Seitz filtered.
6. Lyophilized sera redissolved and Seitz filtered.
7. Purified fractions of sera, Seitz filtered.
8. Lyophilized sera extracted with Bloor's mixture, redissolved in saline and Seitz filtered.

In regard to the extracted sera the procedure was as follows:

Lyophilized serum was extracted with Bloor's mixture (absolute ethyl ether 3 parts and absolute ethyl alcohol 1 part by volume) using 100 ml. of extractant to each gm. of dried protein. The suspension of protein in extractant was shaken 1 hour in the shaking machine and then allowed to settle out overnight in the refrigerator. Next day the clear supernatant was siphoned off and a new lot of extractant was added, to be followed by a similar shaking and settling period. Three such extractions were carried out followed by the collection of the suspended protein on filter paper and its rapid drying under vacuum. When all the Bloor's mixture had

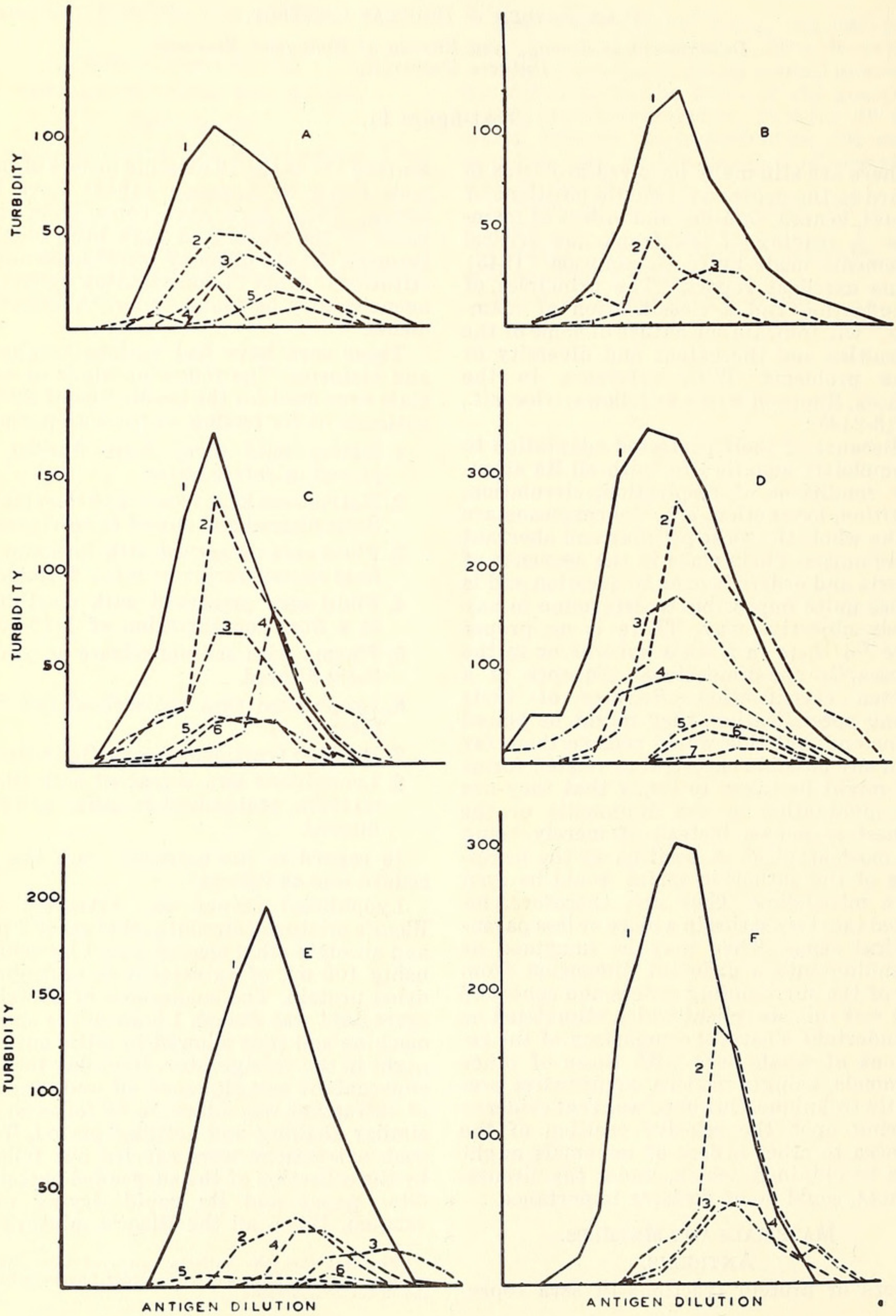
[1] The fact that the antisera employed were made in rabbits automatically removes the order Lagomorpha from the direct comparisons.

been removed the dried protein was redissolved in saline and Seitz filtered.

The purified bovine albumin and fraction II of bovine globulin were obtained from the Armour Laboratories and made up in solutions of appropriate strength for use, viz., about 5%.

ANTISERA.

Sixty-five antisera representing 14 mammalian orders have provided the foci from which a many-sided view of the systematic position of the whales was obtained. All of these antisera were produced in rabbits and most of them by a "presensitization" method. In this procedure a rabbit is given a single injection of 1 ml. of some serum or other antigen, intravenously or subcutaneously, followed by a rest period of 3 or 4 weeks. Thereafter a series of 4 subcutaneous injec-



tions of antigen follows, 1 ml. being given on each alternate day. Seven days after the last injection a trial bleeding is made, and if the antiserum is sufficiently powerful the rabbit is bled out rapidly by cardiac puncture with a 50 ml. syringe, the following day. Food is withheld for 24 hours prior to the bleeding. The blood is allowed to clot in small tubes, kept at room temperature for an hour or two and then placed in the ice chest. Centrifuging may follow later the same day or the next day. The clear antiserum is Seitz filtered and stored in the refrigerator.

If the first series of injections did not give a sufficiently powerful antiserum, additional series may be given. In the case of bovine albumin, alum precipitation was regularly practiced. By these procedures powerful broad-ranging antisera were usually obtained, suitable for the interordinal comparisons.

Testing Procedures

The methods used in making the precipitin tests have been described by Boyden & DeFalco (1943). The validity of such testing procedures involving the Libby Photronreflectometer has been further demonstrated in the reports of Boyden, Bolton & Gemeroy (1947) and Bolton, Leone & Boyden (1948).

Results of the Comparisons.

Table I with its various subdivisions shows the results of the comparisons between the Artiodactyla and Cetacea. Text-figure 1 shows graphically a small but representative selection of some of the curves resulting from the tests bearing upon the relations of the Artiodactyla and Cetacea with each other and with other orders also. The table is subdivided in accordance with the nature of the antigens and antisera and most of the tests are corresponding in the sense

Text-Figure 1. The reaction of six antisera with a variety of antigens.
In each graph the relative turbidities as measured with the Libby Photronreflectometer are plotted along the ordinates and the successive antigen dilutions are plotted along the abscissas. The zone of antigen excess lies in all cases to the left of the peaks of the curves. Each successive antigen dilution contains half the concentration of its immediate predecessor. The relative areas under the curves are determined by summing the ordinates, and the homologous area is the standard of reference for each graph, rated at 100 per cent.

		Area	Per cent.
Graph A Anti-Fin Whale CFo (No. 213)			
Tested with	(1) Fin Whale CFo	579	100
	(2) Sperm Whale 48-3Fo	214	37
	(3) Sperm Whale 48-2Fo	147	25
	(4) Beef R4Fo	85	15
	(5) Pig 45-3CFo	72	12
Graph B Anti-Fin Whale CFo (No. 214)			
Tested with	(1) Fin Whale CFo	519	100
	(2) Beef R4Fo	149	29
	(3) Lyo-beef 47-6Fo	120	23
Graph C Anti-lyosheep 47-1 (No. 209)			
Tested with	(1) Lyosheep 47-1	647	100
	(2) Lyobeef 47-6	457	71
	(3) Giraffe 43-1	294	45
	(4) Beef Albumin 47-1	247	38
	(5) Fin Whale CFo	109	17
	(6) Pigmy Sperm Whale 48-1ME	95	15
Graph D Anti-lyobeef (No. 201)			
Tested with	(1) Lyobeef 47-3	1904	100
	(2) Beef Albumin 47-1	1285	67
	(3) Pigmy Sperm Whale 48-1ME	721	38
	(4) Fin Whale CFo	528	28
	(5) Coyote Ro78	160	8
	(6) Rhesus II	145	8
	(7) Elephant Goss 19	39	2
Graph E Anti-ext. Fin Whale 48-9 (No. 197)			
Tested with	(1) Fin Whale 48-9	853	100
	(2) Lyobeef 48-1	130	15
	(3) Beef Albumin 45-1	81	9
	(4) Kudu 48-1	92	11
	(5) Hedgehog 19-8-48	37	4
	(6) Giant Pangolin 1011	14	2
Graph F Anti-ext. Beef 47-1Fo (No. 233)			
Tested with	(1) Ext. Beef 47-1Fo	1906	100
	(2) Beef Albumin 47-1Fo	674	35
	(3) Fin Whale CFo	284	15
	(4) Pigmy Sperm Whale 48-1MEFo	333	17

TABLE I.

Summary of Values: Artiodactyla vs. Cetacea.

A. ANTINATIVE ARTIODACTYL SERUM × NATIVE WHALE SERUM.

Antiserum	Kind	Homologous Area	Heterologous Area	Kind	Per cent. Heterol. Area / Homol. Area
41	Lyobeef[2]	2135	309	Pigmy Sperm 48-1 ME	15
42	Lyobeef[2]	1272	146	Pigmy Sperm 48-1 ME	12
44	Lyobeef[2]	980	64	Pigmy Sperm 48-1 ME	7
45	Lyobeef[2]	883	170	Pigmy Sperm 48-1 ME	19
47	Lyobeef[2]	1624	59	Pigmy Sperm 48-1 ME	4
52	Lyobeef[2]	1745	94	Pigmy Sperm 48-1 ME	5
53	Lyobeef[2]	194	4	Pigmy Sperm 48-1 ME	2
54	Lyobeef[2]	1220	10	Pigmy Sperm 48-1 ME	1
55	Lyobeef[2]	1073	24	Pigmy Sperm 48-1 ME	2
56	Lyobeef[2]	1602	85	Pigmy Sperm 48-1 ME	5
60	Lyobeef[2]	1254	135	Pigmy Sperm 48-1 ME	11
63	Lyobeef[2]	2522	929	Pigmy Sperm 48-1 ME	37[3]
65	Lyobeef[2]	1978	554	Pigmy Sperm 48-1 ME	28[3]
201	Lyobeef[2]	1904	721	Pigmy Sperm 48-1 ME	38[3]
202	Lyobeef[2]	1383	83	Pigmy Sperm 48-1 ME	6
209	Sheep	647	95	Pigmy Sperm 48-1 ME	15
210	Sheep	898	0	Pigmy Sperm 48-1 ME	0
228	Warthog	1708	240	Pigmy Sperm 48-1 ME	14
229	Warthog	1055	34	Pigmy Sperm 48-1 ME	3
255	Kudu	2085	159	Pigmy Sperm 48-1 ME	8
256	Kudu	1595	218	Pigmy Sperm 48-1 ME	14
				Average	8.9%

[2] The scientific names corresponding to the common names which appear in the table are as follows:

Beef	*Bos taurus*
Sheep	*Ovis aries*
Warthog	*Phacochoerus* sp.
Kudu	*Tragelaphus kudu*
Bison	*Bison bison*
Pig	*Sus scrofa*
Pigmy Sperm Whale	*Kogia breviceps*
Fin Whale	*Balaenoptera physalus*
Sperm Whale	*Physeter catodon*

[3] These values, unusually high for even the inordinal tests of Artiodactyla and Cetacea, have not been included in the average. Further comment regarding them is to be found under the section dealing with the effect of proportion of anti-albumins to total anti-body on the relationship values.

that antinative sera are tested with native sera, or antiformolized sera are tested with formolized sera. The average of all tests in Table I was 10.9%, a substantial value for interordinal tests, to be compared with the value of 1.9% for all other interordinal comparisons made.

Examining the subdivisions of Table I we find that the average value for the comparisons involving native sera was 8.9% (excluding the 3 values for antisera #63, 65, 201, which are treated separately); for the comparisons involving formolized antigens it was 13.2%; for the comparisons involving antisera to extracted antigens it was 9.0%; and, finally, for the comparisons involving antisera to particular bovine fractions it was 8.2%.

Out of the 65 sets of curves available, a small sample involving six antisera is shown in Text-figure 1. All the curves belong to the same general type; in fact this is true regardless of the kind of antigen and antiserum used.

The first tests in this investigation were made with the antisera shown in the upper two graphs (A & B) of Text-figure 1. These were anti-fin whale sera. The first heterologous tests made with these antisera were with beef serum and the surprisingly strong reactions gave us the clue which has been followed to a logical conclusion. These tests were made with antisera to formolized antigens tested with formolized antigens.

Charts C and D of Text-figure 1 show the results of testing antisera made to lyophilized sera. In chart C, an antisheep serum was tested with sheep, beef and giraffe sera, and with beef albumin, to show the magnitudes of the artiodactylan reactions in comparison with those of the cetaceans. "Fin whale C" serum was available only as formolized antigen.

Graph D illustrates the results of testing an antibeef serum with a variety of antigens of several orders. This antiserum contained an unusually high proportion of anti-albumins and gives generally higher heterologous values than do antisera with lower relative amounts of anti-albumins.

Graphs E and F illustrate the reactions obtained with antisera made to extracted antigens. E shows the results of testing an anti-fin whale serum with representatives of the Artiodactyla, Insectivora and Pholidota. F shows the results of testing antibeef serum made to extracted and formolized beef serum with formolized whale sera and to formolized beef albumin. The cetacean-

TABLE I (continued).

B. ANTIFORMOLIZED SERA × FORMOLIZED SERA.

Antiserum	Kind	Homologous Area	Heterologous Area	Kind	Per cent. Heterol. Area / Homol. Area
213	Fin Whale C Fo	579	85	Beef R4 Fo	15
	C Fo	579	35	Bison Ro17 Fo	6
	C Fo	579	72	Pig 45-3c Fo	12
214	C Fo	519	149	Beef R4 Fo	29
	C Fo	519	120	Lyobeef 47-6 Fo	23
	C Fo	519	32	Bison Ro17 Fo	6
	C Fo	519	23	Longhorn Ro18 Fo	4
	C Fo	519	49	Pig 45-3c Fo	9
234	Lyobeef Fo	1789	309	Pigmy Sperm 48-1 ME Fo	17
	Lyobeef Fo	1789	205	Fin Whale C Fo	11
				Average	13.2%

C. ANTI-EXTRACTED SERA × NATIVE OR FORMOLIZED SERA.

Antiserum	Kind	Homologous Area	Heterologous Area	Kind	Per cent. Heterol. Area / Homol. Area
197	Ext. Fin Whale	853	130	Lyobeef 48-1	15
	Ext. Fin Whale	853	92	Kudu 48-1	11
	Ext. Fin Whale	853	0	Warthog Goss 47-Z2	0
198	Ext. Fin Whale	918	60	Lyobeef 48-1	7
199	Ext. Fin Whale	835	119	Lyobeef 48-1	14
205	Ext. Lyobeef	753	44	Pigmy Sperm 48-1 ME	6
206	Ext. Lyobeef	2040	158	Pigmy Sperm 48-1 ME	8
211	Ext. Lyosheep	1360	43	Pigmy Sperm 48-1 ME	3
212	Ext. Lyosheep	1454	31	Pigmy Sperm 48-1 ME	2
233	Ext. Beef Fo	1906	333	Pigmy Sperm ME Fo	17
233	Ext. Beef Fo	1906	294	Fin Whale C Fo	15
				Average	9.0%

D. ANTIBOVINE FRACTIONS × NATIVE OR FORMOLIZED WHALE SERA.

Antiserum	Kind	Homologous Area	Heterologous Area	Kind	Per cent. Heterol. Area / Homol. Area
2	Anti-albumin	1573	92	Fin Whale C Fo	6
246	Anti-albumin	2239	409	Pigmy Sperm 48-1 ME	18
257	Anti-albumin Fo	1022	107	Pigmy Sperm 48-1 ME Fo	10
257	Anti-albumin Fo	1022	50	Fin Whale C Fo	5
3	Globulin II	779	0	Fin Whale C Fo	0
237	Globulin II	1307	133	Pigmy Sperm 48-1 ME	10
				Average	8.2%

artiodactylan relationships shown in graphs E and F are typical values.

The data given in the table and graphs serve as the basis for the following tentative conclusions:

(1) The tests involving formolized antigens, and antisera made to them, appear to give somewhat higher average values than the remaining tests.

(2) The values obtained with extracted antigens and antisera made to them, or with purified fractions and their corresponding antisera, are not significantly different from the values based on native comparisons. There is no evidence therefore that lipoidal materials have affected the values significantly.

(3) Of the values obtained with purified antigens shown in Table I, D, those based on the albumins give a higher average (10%) than those based on the globulins (5%).

(4) There is considerable variability in the individual values shown throughout, a fact which needs further analysis and explanation.

DISCUSSION.

Some explanation of the variability of results obtained when different antisera of the same kind are tested is needed. Every rabbit responds as an individual to the treatment with an antigen, and produces antibodies of amounts and kinds in accordance with its inherent physiological capacity. The antisera so produced by different rabbits may vary (1) in their strength, i.e., their antibody content; (2) in their sensitivity, i.e., in their capacity to react with minimal amounts of antigen; (3) in their range, which is the capacity to react with distantly related or chemically diverse antigens; (4) in their specificity, which is their capacity to react differentially with a variety of antigens; and (5) in the numbers and proportions of the different kinds of antibody produced to serologically distinct antigens. With such possibilities of variation among antisera of the same kind, i.e., produced to the same composite antigen, the amazing thing is the relatively great consistency of the results, for some rabbits were given but a single injection series, and others two or three injection series, following the original presensitization. That this variation in injection procedure affects the range and specificity of the reactions with antisera made to mammalian sera is probably true.

In addition to other causes of variability in the relationship values than those hitherto discovered, we find that the proportion of anti-albumins to total antibodies in the antiserum seems to be highly significant. Thus the three antisera listed in Table I, A, with highest relationship values (#63, 65 and 201) belong in a class by themselves, having 67 to 70% of anti-albumins, whereas the anti-albumin content of 16 other antibovine sera varied from 0 to 45% with an average of 20%. Again, the anti-albumin tests in Table I, D, gave an average value which was about double the value for the globulin II tests. These data may be interpreted to mean that the albumins of the Artiodactyla and Cetacea are more similar than their globulins and have been more conservatively evolved. If this conclusion is justified in these comparisons, it may hold for mammalian comparisons in general, and our preliminary tests indicate that this is true. If so, an important additional source of variability in the magnitude of relationship values has been discovered, to be reckoned with in the future. It may well be that the anti $\frac{A}{G}$ ratio for antisera produced to vertebrate sera, which are mixed antigens, will become as significant in systematic serology as the $\frac{A}{G}$ ratio is in general physiology. The analysis of the anti-albumin content of these 19 anti-bovine sera was made relatively easy by the availability of purified bovine albumins obtained through the Armour Laboratories.

The order of magnitude of some of the tests involving the Cetacea and other orders of mammals except the Artiodactyla is shown in the text-figure. The average of 59 such tests made with antisera to 13 orders and antigens representative of 16 orders of mammals was 2.1% .These interordinal tests involved the Cetacea compared with one or more representatives of the Monotremata, Marsupialia, Insectivora, Chiroptera, Dermoptera, Primates, Edentata, Pholidota, Rodentia, Carnivora, Tubulidentata, Proboscidea, Hyracoidea, Sirenia and Perissodactyla.

The average interordinal value of 2.1% between the Cetacea and other orders, except the Artiodactyla, is of the same order of magnitude as the average value of interordinal tests between the Artiodactyla and the other orders exclusive of Cetacea, or among a variety of other orders excluding Artiodactyla and Cetacea. This average value was 1.6%, involving 44 tests and 15 orders. Of course the Lagomorpha cannot be placed in these interordinal comparisons because the antisera were all produced by rabbits. It is true that some orders were necessarily represented by single species, because additional species or samples were unavailable. It is true also that not as many antisera were made to the orders other than Artiodactyla and Cetacea as to these two orders, but there has been no hint of values consistently high enough to warrant further testing.

Taken as they are the data justify the conclusion that the serum proteins and especially the albumins of Artiodactyla and Cetacea are more similar than the corresponding proteins of the other orders tested, and presumably therefore the Cetacea and Artiodactyla have a closer blood and genetic relationship than the other orders do. The significance of these conclusions may be discussed for some time to come.

The various conflicting views and interpretations of the morphological evidences regarding cetacean relationships are presented in full to that time in Kellogg's report, "A Review of the Archaeoceti" (1936). Kellogg tentatively concludes that the Cetacea have descended from some primitive insectivore-creodont ancestry of long ago, admitting the inadequacy of the morphological evidences to settle the question. As previously stated, Simpson (1945) finds the evidences too uncertain to permit of any definite placement of the Cetacea. The report of H. W. Mossman (1937) on "Comparative Morphogenesis of the Fetal Membranes and Accessory Uterine Structures" places the Cetacea and Artiodactyla together as derivatives of a primitive ungulate stock. This interpretation accords very well with ours based on the blood tests.

We have provided evidence (Boyden, 1942; Boyden, Gemeroy & Bolton, 1949) that the serum tests accord with genetic relationships where they are known, as in the relation of mule and hinny to horse and ass. There is also considerable evidence that precipitin tests accord well with systematic relationships among close relatives or where the systematic positions are fairly well established. This is true for Crustacea (Boyden, 1943) Insecta (Leone, 1947 a and b) Pisces (Gemeroy, 1943) Amphibia (Boyden & Noble, 1933) Aves (DeFalco, 1942) and Mammalia (Boyden, 1926, 1942), (Boyden & DeFalco, 1943).

In such a problem as that of the systematic position of the whales, where the morphological evidences are inconclusive and inadequate, other sources of information are needed. Under these conditions the serological reactions may fill this need and provide evidence of a decisive nature.

Summary.

Powerful precipitating antisera have been used to test the serological relationships of the Cetacea to the other orders of mammals. The interordinal reactions as determined by the quantitative photoelectric technique using the Libby Photronreflectometer are generally weak, averaging about 2%, with the exception that the artiodactyl-cetacean

comparisons are distinctly higher, averaging about 11%. The tests therefore indicate that there is a greater similarity in the serum proteins of representative Cetacea and Artiodactyla than between the Cetacea and any other orders tested, all existing orders but the Lagomorpha being included in the comparisons. Furthermore the tests indicate that there is a relatively greater similarity of the serum albumins of these two orders than of their globulins. The conclusions are therefore drawn that the Cetacea should be granted a greater degree of systematic relationship to the Artiodactyla than to any of the other mammalian orders tested and that the albumins of the sera of these two orders have been more conservatively evolved than their globulins.

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