On the Precipitin Reaction in Sea Anemones, as Examined by the Ring Test Method[1]

By

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(With 1 Text-figure and 3 Tables)

Ever since the classical work by Portier and Richet (1902), it has long been known that a dog, hypersensitized by injecting an extract of sea-anemone, responds with an anaphylactic shock to a reinjection of the same extract. The anaphylaxy having been determined as a kind of serological reaction, it is not unusual to expect that the precipitin, besides the anphylactin, is reproduced in the animal under test. In fact, from the writer's tentative examination, the specific precipitin reaction became evident in the blood of the dog and of a rabbit, after both had been sufficiently immunized to sea-anemone extracts by repeated injections. Also, in regard to the production of precipitin, the rabbit was found to be more serviceable after tests than the dog. To study the serological differentiation and relationship of some sea-anemone species, these experiments were made, using a precipitin reaction on the rabbit antisera.

Before going further, the writer wishes to express his cordial thanks to Prof. Dr. T. Uchida, who has given many valuable suggestions during the course of the present work and helped to identify the species reported herein. Also to Dr. Y. Watanabe, Professor of Physiological Zoology of the University of Ibaraki, acknowledgement is due for his helpful criticisms and assistance in the preparation of this manuscript.

Material and Method

The sea-anemone antigens used in the present study were procured from the following four species, all of which were collected from the coast of Akkeshi,

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Hokkaido : *Anthopleura xanthogrammica* Brandt, *Epiactis prolifera* Verrill, *Tealia felina* var. *coriacea* Rapp and *Metridium senile* var. *fimbriatum* Verrill.[1]

The antigens and antisera were prepared by applying the principles introduced by Wilhelmi (1940). To clear out debris and foreign proteins, contained in the gastric cavity, or stuck on the body surface, the sea-anemones were kept in clean running sea-water without food from one to two weeks. The animal body was well minced and ground in a mortar. For the removal of lipoid substance, the ground material was treated with the mixture of three parts of absolute alcohol and one part of ethyl ether, for 2 to 5 hours, at the temperature from 0° to 5°C Then, the material was immediately dried in a vacuum desiccator and stored in a dried condition until needed. Antigen was extracted from the dried material with a physiological saline solution (0.8 per cent NaCl at pH 7.4). The amount of antigen was denoted by the amount of dry weight of antigenic material in the solution. For the preparation of rabbit sera immune to sea-anemone antigens, antigen solution was injected, every two days, into a lateral ear vein of an adult rabbit, but with a successive increase of dosage, viz., 4, 8, 12 and 16 mg. Ten days after the 4th inoculation, the whole blood was shed out from the carotid artery of the rabbit ; then antiserum was separated from the blood clot and inactivated by heating about half an hour in a water bath of 56°C. The antiserum with a little carbolic acid (0.5 per cent, at most) as preservative was prepared and stored in a cold room at a temperature of 0° to 5°C.

In regard to the injection of sea-anemone extracts, it should be noted that "actinocongestin", a nematocyst toxin, would give a certain rise to the anaphylactic shock in the animal. In fact, the antigen from *T. felina* var. *coriacea* caused intense shock sometimes resulting in death to the rabbit, when reinjected. Therefore, an attempt was made to desensitize the rabbit by applying a preliminary hypodermic injection of 1 to 1.5 mg of antigen, before the afore mentioned dosages were intravenously applied. Even after this precaution, a certain degree of anaphylactic manifestations could not be prevented, when a single dose of antigen injection exceeded 10 mg. For this reason, the dosage of antigen to be applied was subdivided into smaller amounts, each of which was repeatedly injected at intervals of about one hour, until the amount added up to the total required.

In these tests, the precipitin reaction was examined by three stages : alpha- and beta-ring tests and absorption test. In view of the current general opinion that titer of the ring tests indicates the end point of the complete interaction between antigen and antibody, the reading of these tests was taken after 5 to 6 hours of incubation at room temperature (12° to 15°C). In such long a period of incubation, the ring formed, but of course, became diffused and stagnant ; therefore, it was not satisfactory to determine the exact end point of the reaction. In order to

1) In the following pages, only generic names are used for the sake of descriptive convenience.

overcome this difficulty as far as possible, the reaction in each vial of serial dilutions was continuously observed during the whole course of incubation, in which the formation of the ring gradually took place. In most cases, the titer thus obatined for each individual test did not vary over a range of from 1/2 to 2 times in dilution from the average. The antigen dilution taken as an original was 1 : 200, i. e., 1 gram of the soluble antigen in 200 cc of the physiological saline solution. In each series, starting with this amount, the antigen solution was serially diluted by doubling the volume with saline.

Experimental Results

Alpha-ring test (antigen titers) : For the examination of serological differentiation among the sea-anemones, alpha-ring test was first applied with undiluted antisera. The data obtained are given in Table. 1.

Table 1. Antigen titers against undiluted antisera

Antiserum	Antigen			
	Anthopleura	Epiactis	Tealia	Metridium
Anthopleura	1 : 102400 +	1 : 102400	1 : 51200 +	1 : 12800 +
Epiactis	1 : 204800	1 : 204800	1 : 102400 ±	1 : 51200
Tealia	1 : 51200 ±	1 : 102400	1 : 102400 +	1 : 51200
Metridium	1 : 25600	1 : 51200	1 ; 102400	1 : 102400 +

+ represnts 'slightly positive', and ±, 'quite traceable' in reaction with next higher antigen dilution.

As shown in Table 1, the homologous titers with the corresponding antisera are constant and estimated as 1 : 102400, except in *Epiactis*, in which they are 1 : 204800. The heterologous titers in each reciprocal tests fairly well generally coincide with each other, but with a few exceptions. As to the cross reactions, the titer of *Anthopleura* antigen is high with anti-*Epiactis* serum, next higher with anti-*Tealia* serum and low with anti-*Metridium* serum ; and, with anti-*Anthopleura* serum it is highest in *Epiactis* antigen, low in *Tealia* antigen, and still lower in *Metridium* antigen. Briefly speaking, the alpha-ring test titer for *Anthopleura*, acording to the data given in Table 1, decreases in the following order : *Epiactis*>*Tealia*>*Metridium*. Similarly, in three other species the titer value of alpha-ring test may be arranged in the following series :

For *Epiacits* : *Anthopleura*≧*Tealia*>*Metridium*,
For *Tealia* : *Epiactis*≧*Metridium*≧*Anthopleura*,
For *Metridium* : *Tealia*≧*Epiactis*>*Anthopleura*

For the determination of their relationship, however, the degree of difference

shown by the data is not so distinct as compared with titer variation for each individual test. Moreover, the orders arranged above appear too irregular to draw any definite conclusion from these series. What might be permissible on the basis of the available results seems only to suggest that, among the species here examined, *Anthopleura* and *Metridium* are serologically most scarcely related with each other.

Beta-ring test (*antiserum titers*) : Attacking the problem of the serological differentiations among different animal phyla, Wilhelmi (1942) employed very strong antisera. For the present investigation, however, such strong antisera are not quite preferable. It is often impossible with too strong antisera to discriminate the homologous antigen from any heterologous antigens from closely related species, since, frequently, the titer appears the same in both reaction. For instance, as shown in Table 1, with undiluted anti-*Epiactis* serum the homologus (*Epiactis*) antigen has given the same titer as the heterologous *Anthopleura* antigen (line 3). Nevertheless, it remains to be seen whether they have the same capacity for antigenecity. Thereafter, for such closely related species as the sea-anemones to be examined here, the ring test titers of both, homologous and heterologous, were determined with use of various dilutions of antisera. The results are summarized in Table 2.

From the tabulated data, it is observed that highest dilutions of both antigens and antisera, with which the positive precipitin reaction is permitted, differ in greater or less degree, according to different combinations of antigen and antiserum.

Upon examining of the highest dilutions (antiserum titers) of anti-*Anthopleura* serum and anti-*Epiactis* serum with homologous and heterologous antigens from both species, species-specificities were not shown by anti-*Anthopleura* serum, but distinctly shown by anti-*Epiactis* serum (columns 2 to 5 of Table 2). Similarly,

Table 2. Antigen Titers and

Dilution of antiserum	Combination of antigen				
	Anti-Anthopleura serum		Anti-Epiactis serum		
	Anthopleura antigen	Epiactis antigen	Anthopleura antigen	Epiactis antigen	Tealia antigen
1 : 2	1 : 102400 +	1 : 102400	1 : 204800	1 : 204800	1 : 102400 ±
1 : 4	1 : 102400 +	1 : 102400	1 : 102400	1 : 204800	1 : 102400
1 : 8	1 : 102400 +	1 : 102400	1 : 102400	1 : 204800	1 : 102400
1 : 16	1 : 102400 +	1 : 102400	1 : 102400	1 : 204800	1 : 102400
1 : 32	1 : 102400	1 : 102400	1 : 102400	1 : 204800	1 : 51200
1 : 64	1 : 25600 +	1 : 25600 +	1 : 51200	1 : 102400	1 : 12800 +
1 : 128	1 : 12800	1 : 12800	1 : 12800 +	1 : 102400	1 : 6400 +
1 : 256	0	0	0	1 : 12800	0
1 : 512	0	0	0	1 : 12800	0
1 : 1024	0	0	0	0	0

0 represents 'negative' in reaction with antigen of the original dilution (1 : 200), and

antigens from *Epiactis* and *Tealia* were easily distinguishable with use of anti-*Epiactis* serum, while the reciprocal reactions with anti-*Tealia* serum have shown no difference in titer between the two (columns 5 to 8). The data in columns 4 to 6 of Table 2 are graphed in Figure 1, in which logarithms of 1/100 antigen titers are given as ordinates, and logarithums of dilutions of antiserum as abscissae, on the scale of a unit to log 2. In the light of these graphs, it may be concluded at a glance that antigen from *Epiactis* seems to contain nearly all the components specific to *Anthopleura* and *Tealia*.

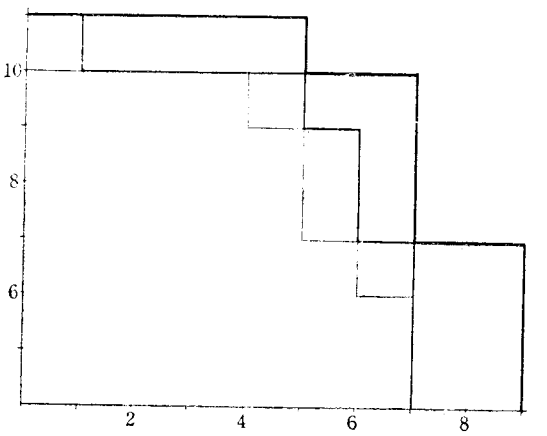

Fig. 1. Graphs showing the relationship of titers of antigens from *Anthopleura*, *Epiactis* and *Tealia* to dilutions of anti-*Epiactis* serum. Ordinates and abscissae are difined in the text. Heavy line from the data on *Epiactis* antigen, medium line from the data on *Anthopleura* antigen and light line from the data on *Tealia* antigen. These graphs are based on the data in columns 4 to 6 of Table 2.

In regard to the relation between *Tealia* and *Metridium*, the com-

Antiserum Titers

and antiserum

Anti-Tealia serum			Anti-Metridium serum	
Epiactis antigen	Tealia antigen	Metridium antigen	Tealia antigen	Metridium antigen
1 : 102400	1 : 102400 +	1 : 51200	1 : 102400	1 : 102400 +
1 : 102400	1 : 102400	1 : 51200	1 : 102400	1 : 102400 +
1 : 102400	1 : 102400	1 : 51200	1 : 51200 +	1 : 102400 +
1 : 102400	1 : 102400	1 : 25600	1 : 51200	1 : 102400
1 : 25600	1 : 25600	0	1 : 25600	1 : 102400
1 : 12800	1 : 12800	0	0	1 : 51200
1 : 6400	1 : 6400	0	0	1 : 3200
0	0	0	0	0
0	0	0	0	0
0	0	0	0	0

other marks are as denoted in Table 1.

parison of highest dilutions of reactive antisera (columns 8 to 11) makes it readily possible to discriminate with each other, since they are unquestionably higher in homologous reactions than in heterologous reactions, irrespective of the species of antiserum used. And from the comparison of the data on *Anthopleura*, *Epiactis* and *Tealia* in the columns 2 to 8 with those on *Tealia* and *Metridium* in the columns 8 to 11, it appears, in general, that serological difference is much greater between *Tealia* and *Metridium* than those among *Anthopleura*, *Epiactis* and *Tealia*, and, particularly, that *Tealia* is more closely related to *Epiactis* than to *Metridium*. Accordingly, taking the data shown in Table 1 into account, it seems highly probable that *Metridium* is serologically more or less independent of the group of other three species.

Absorption test: Usually it is considered that the specific reaction of antiserum with its homologous antigen is generally well established by means of partial absorption with the heterologous antigens at the equivalence point of the given reaction system. It is regretable, however, that the scantiness of antisera provided in the present study obliged the writer to experiment without being able to determine the equivalence point of each system. In this investigation, the antigens were discretionally diluted with 0.8 per cent NaCl solution, dissolving 1 gram of gum arabic per 100 cc. Equal volumes of antigen and antiserum solutions were mixed, and incubated for 2 hours at the temperature of 37°C. Then the mixture was

Table 3. Titers in absorption test

Immune serum	Absorbed by extracts of	Titers of precipitations with antigen of			
		Anthopleura	Epiactis	Tealia	Metridium
Anthopleura (1 : 2)	*Anthopleura* (1 : 3200)	1 : 25600±	1 : 25600		
	**Anthopleura* (1 : 400)	1 : 6400	0		
Anthopleura (1 : 2)	*Epiactis* (1 : 3200)	1 : 25600	1 : 25600		
	**Epiactis* (1 : 400)	1 : 12800	0		
Epiactis (1i: 4)	*Epiactis* (1 : 1600)	1 : 6400	1 : 25600		
Epiactis (1 : 2)	*Anthopleura* (1 : 3200)	1 : 25600	1 : 204800		
Epiactis (1 : 4)	*Tealia* (1 : 3200)		1 : 102400	1 : 12800	
Tealia (1 : 2)	*Epiactis* (1 : 1600)		1 : 25600	1 : 25600	
	**Epiactis* (1 : 800)		0	1 : 6400	
Tealia (1 : 2)	*Metridium* (1 : 3200)			1 : 51200+	1 : 12800
Metridium (1 : 2)	*Tealia* (1 : 3200)			1 : 12800	1 : 51200

* represents 're-absorption', and other marks are the same as in Tables 1 an 2.

kept overnight at 5° below zero in C. The precipitate being removed by centrifuging after each admixture, the supernatant was employed for the test. Table 3 show the results obtained.

If, as suggested above, *Epiactis* antigen contains almost all the antigenic components specific to *Anthopleura*, it would be expected to be able to distinguish *Epiactis* from *Anthopleura* with use of the specified anti-*Epiactis* serum, but unable to do so with anti-*Anthopleura* serum. This has been proven by the data presented here. When anti-*Anthopleura* serum (1 : 2) has been absorbed with the antigen (1 : 3200) from *Anthopleura* or from *Epiactis*, the homologous titers are found to be exactly the same as the heterologous titers (1 : 25600) (lines 3 and 5 of Table (3). On the contrary, when the anti-*Epiactis* serum (1 : 2) has been absorbed with *Anthopleura* antigen (1 : 3200), the homologous titer is rather high (1 : 204800) as compared with the heterologous titer of *Anthopleura* antigen (1 : 25600) (line 8). Even when the anti-*Epiactis* serum (1 : 4) has previously been absorbed with *Epiactis* antigen of low dilution (1 : 1600), the titer of *Epiactis* antigen is higher than that of the *Anthopleura* antigen (line 7). However, the anti-*Anthopleura* serum can be employed for the discrimination of the antigens between the two species, if it has been exhausted again with much less diluted antigen from *Anthopleura* serum, or from *Epiactis* (lines 4 and 6). Against such highly exhausted anti-*Anthopleura* serum, the reaction appears still positive with *Anthopleura* antigen, while it no longer positive even with *Epiactis* antigen of the original dilution (1 : 200) (lines 4 and 6).

Similar serological relation has been found between *Epiactis* and *Tealia*. That is to say, *Epiactis* antigen can readily be distinguished from *Tealia* antigen with use of absorbed antiserum of the former, but the reverse is hardly the case (lines 9 to 11). From the data on *Tealia* and *Metridium* in lines 12 and 13 of Table 3,it is also quite evident that, with antiserum (1 : 2), which has been absorbed with heterologous antigen (1 : 3200), the homologous titer is always higher (1 : 51200) than the heterologous titer (1 : 12800), no matter which of the two antisera may be used (line 12 and 13).

Discussion

In the recent progeress in the precipitation technique, the turbidimetric method, using a photronreflectometer, appears to be more advisable than the ring test method.[1] However, unfortunately, at this writing, the instrument was not yet placed at the disposal of the writer. Thus, the preceeding examination of the sea-anemones were conducted with the ordinary technique of alpha- and beta-ring tests and the absorption method, but with utmost care so far as possible.

As the preceeding data tend to point out, *Anthopleura*, *Epiactis* and *Tealia*

1) Ogata, Matsubayashi and Suzuki, 1938; Matsubayashi, 1938; Boyden and DeFalco, 1943; Bolton, 1947; Bolton, Leone and Boyden, 1948; etc.

are in serological sense rather intimately related with one another, but *Epiactis* antigen is easily distinguishable from that of *Anthopleura* as well as *Tealia* with use of diluted anti-*Epiactis* serum, and not possible with anti-*Anthopleura* and anti-*Tealia* sera. This may indicate that the antigenic constitution of *Epiactis* is consisted of more complex components than the antigen from *Anthopleura* and *Tealia*, but specially contains nearly all of them. Similar relation has been found by several workers in higher animals, e. g., in Japanese ducks, wild and domestic (Sasaki, 1928), rabbit and hare (Moribe, 1928), goldfish and crucian (Ishihara and Misao, 1929), and in wild boar and domestic pig (Sasaki and Moribe, 1930). The results obtained by them all agree in showing that the antigen of the domestic animal is much more complex than in the wild form. Moreover, Moritz (1934), and in more recent year Irwin (1947) has proved the fact that the antigenic constitutions of the hybrids are represented by combinations of all, or parts of antigenic components from their parent species, without (Moritz), or with or without (Irwin) the occurrence of specifically new component as the results of hybridization. With regard to this point, the data on the reciprocal cross reactions among *Anthopleura*, *Epiactis* and *Tealia* appear to throw some light to the genealogical problem of these species.

According to the taxonomic investigation by Stephenson (1935), *Anthopleura*, *Epiactis* and *Tealia* are included in the same family, Actiniidae, while *Metridium*, in a different family, Metridiidae. As stated above, *Metridium* is found to be serologically somewhat apart from the group of other three species. In consequence, in so far as these four species are concerned, the family affiliation has been proven to demonstrate with serological method. As to the rank of each species in the zoological system, however, the writer wishes to reserve any difinite conclusion on the basis of systematic serology, until more extensive data have been obtained.

In connection with the data presented in the foregoing tables, there should be some mention of the work of Boyden and DeFalco (1943), in demonsrating the increase in the capacity of antiserum for discriminating the antigens from the closely related species, by means of dilution of the antiserum. From the data given in Tables 1 to 3, it is clear that the undiluted anti-*Epiactis* serum has almost no capacity for discriminating homologous (*Epiactis*) antigen from heterologous *Anthopleura* antigen, but seems to be capable when diluted, or absorbed beforehand with homologous antigen. It is probable that, in anti-*Epiactis* serum, dilution and partial absorption may bring about the similar effect as to increase in capacity for discrimination of the antigens from the intimately correlated species. However, upon testing the antiserum titers (Table 2), no effect of dilusion could be seen on the discriminating capacity in anti-*Anthopleura* serum and anti-*Tealia* serum. Accordingly, Boyden and DeFalco's principle should not be widely applicable without limitation, at least in the ring test method. The practical application of this principle appears to be dependent on a certain definite difference or differences

in the effect of dilution upon the reactivity of antibody components, either species-specific or non-species-specific to the animal. If the above argument is admitted, when there is no appreciable difference in effect of dilution upon the antibody components in question, thus this method must be of no use for discrimination of the species. In such cases, the method of partial absorption appears to be more preferable, if the precipitin reaction is examined by the ring test method. Needless to say, for the verification of this principle of Boyden and DeFalco with these sea-anemone species, a further attempt is necessary to compare the precipitate amounts in heterologous reaction between *Epiactis* antigen and anti-*Anthopleura* or anti-*Tealia* serum of various dilutions with those in the homologous reactions with the antisera of the corresponding dilutions.

Summary

For the study of serological differentiation and relationship of four species of sea-anemone, the precipitin reaction was examined by the ring test method. The data presented reveal first, that *Anthopleura xanthogrammica* and *Metridium senile* var. *fimbriatum* are serologically most scarcely related with each other, and second, that *Tealia felina* var. *coriacea* is more closely related to *Epiactis prolifera* than to *M. senile* var. *fimbriatum*, and third, that *E. prolifera* can be readily distinguished serologically from *A. xanthogrammica* and *T. felina* var. *coriacea* with use of diluted anti-*Epiactis* serum, but hardly with anti-*Anthopleura* serum and anti-*Tealia* serum. The antigen of *E. prolifera* appears to be more complex than those of *A. xanthogrammica* and *T. felina* var. *coriacea*, and contains nearly all the antigenic components specific to the latter two. On the whole, the evidence seems to indicate that the serological differentiation and relationship thus observed may conform rather well with the current taxonomic classification of these four species of sea-anemone.

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