Serological studies of moth proteins with special reference to specific immune bodies and their phylogenetic significance
by Saxon Martin

A THESIS Submitted to the Graduate Committee In partial fulfillment of the requirements for the Degree of Master of Science in Botany and Bacteriology at Montana State College
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SEROLOGICAL STUDIES OF MOTH PROTEINS WITH SPECIAL REFERENCE TO SPECIFIC IMMUNE BODIES AND THEIR PHYLOGENETIC SIGNIFICANCE

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Approved:

F. B. Peters
In Charge of Major Work

D. B. Swingle
Chairman Examining Committee

F. B. Peters
Chairman Graduate Committee

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SEROLOGICAL STUDIES OF MOTH PROTEINS WITH SPECIAL REFERENCE TO SPECIFIC IMMUNE BODIES AND THEIR PHYLOGENETIC SIGNIFICANCE

INTRODUCTION

The significance attached to immunological reactions and especially to the so-called specific immune body in immunological reactions of various bacteria has been presented by Krumweide and Noble (12), Heidelberger and Avery (8), Gunnison and Shoenholz (6), Hitchcock (11), Sugg and Neill (20), Lancefield (16), and others. The presence of these bodies in the higher forms has been demonstrated by Landsteiner (15) in his work on horse saliva.

A specific immune body has been isolated from collections of Feltia ducens Wlk. belonging to the family Phalaenidae. The writer has demonstrated that the precipitin reaction can be used to determine a phylogenetic relationship between members of this group.

There has been little work done on the specific immune bodies occurring in members of various phyla of the animal kingdom and no available literature as regards such work on insects.

Krumwiede and Noble (12) described a rapid method for the production of precipitin antigen from bacteria. Heidelberger and Avery (8) described a method used in obtaining the soluble specific substance from Pneumococcus in which they isolated the soluble specific substance by the concentration of broth cultures, precipitation with alcohol, repeated

re-solution and precipitation. This was followed by a careful series of fractional precipitations with alcohol or acetone after acidification of the solution with acetic acid and repeated fractional precipitation with ammonium sulphate and dialysis of the aqueous solution of the active fractions. Their material contained 1.2 per cent of nitrogen and yielded 79 per cent of reducing sugars on hydrolysis. The material gave a specific reaction in a dilution as high as 1:5,000,000.

Landsteiner and Levene (14) obtained an active substance from dried horse kidneys which inhibited hemolysis in high dilutions. Balls (2) found a considerable degree of specificity in identifying certain yeasts with the precipitin test. He suggested that a grouping of nearly related species could be made by this method. The precipitin reaction was found to be highly specific for two types of Bacillus botulinus van Ermengem and for the sub-groups within those types by Gunnison and Schoenholz (6).

Hektoen (10) worked with the precipitin reaction on body constituents and found that serum proteins, hemoglobin, and specific precipitinogen in serum were found to be specific for the species. Landsteiner and Levene (14) working on alcoholic extracts from horse kidneys obtained a water soluble substance which they purified with pyridine, chloroform, and alcohol. The final residue was hydrolyzed with sulphuric acid and the aqueous solution of the product of hydrolysis was titrated with Fehling's solution giving a reduction corresponding to 28 mg. of glucose. Hitchcock (11) in his work on the separation of a serological

group described a method in which he used sodium hydroxide to extract the antigenic substance from streptococci. Ando (1) used a modification of Heidelberger's and Avery's method in working with Streptococcus viridans Schotmuller, Bacillus dysenteriae Shiga, and Bacillus mallei Hoefler and Schutz and suggested its use in the diagnosis of glanders. Enders (5) found a type-specific substance in the autolytic products of Type I pneumococci distinct from the specific carbohydrates. He distinguished it from the carbohydrates by the fact that it was not stable when boiled in weakly alkaline solution. Tillet, Goebel, and Avery (21) worked on the chemical and immunological properties of a species-specific carbohydrate of pneumococci. They suspended the organisms in 50 cc. of saline and broke them down by repeatedly freezing and thawing them. The final solution after treatment with acetic acid and sodium hydroxide contained fraction C which was found to be non-specific.

Sugg and Neill (20) described a method for the extraction of antigens in their studies on the relationships among the pneumococci in which the antigen was prepared by boiling cells in .05 normal HCl and adding acetic acid to precipitate the proteins. The active substance was then precipitated with alcohol.

Landsteiner (15), working on horse saliva, obtained an active substance by treatment with acid and acetone and fractionation with alcohol, which gave a weak biuret reaction and on hydrolysis yielded 48.5% reducing sugar.

Zozaya and Wood (24) found that the polysaccharide and the "nucleoprotein" obtained from different types of meningococci, gonococci, and Micrococcus catarrhalis Pfeiffer, had similar immunological properties and were not type-specific. They also found that the carbohydrate-precipitable substance in immune sera appeared in the fourth month or more in the immunization of animals.

Zozaya (22) showed that polysaccharides can be rendered antigenic by haptogenic adsorption upon a colloid carrier. With the polysaccharide of Type III pneumococci he was unable with his method of immunization of rabbits in six weeks to produce any detectable protective antibodies, but was able to produce anticarbohydrate antibodies. All of the bacterial carbohydrates were non-antigenic when used alone. Zozaya (23) also found that the synthetic polysaccharide produced by Leuconostoc mesenterioides (Cienkowski) Van Tieghem from saccharose, reacted immunologically with antisera from pneumococci, some of the Salmonella and some of the types of Streptococcus viridans Bargen. He says further, "this immunological relationship is independent of the specific antipolysaccharide antibodies of these sera, suggesting the existence of a distinct antibody produced by an active group of the specific bacteria polysaccharide, which is similar or identical to the active group of the dextran polysaccharide." "These findings warrant the generalization that antigens need not be of a complex protein nature, but that some substance of a colloid nature is responsible for antigenicity. The colloid may in certain cases be a protein, but any colloid which can adsorb the specific

principle can suffice. The physical attachment of the specific principle to the colloid carrier makes the combination an antigen. In the case of pure proteins, we no doubt have a special radical in the whole molecule which is the active group and gives the antibody its specificity."

Heildelberger and Kendall (9) found fractions which yielded specific precipitates with Type III antipneumococcus horse serum but failed to precipitate homologous rabbit antisera, giving rise only to specific inhibition.

Lancefield (16) showed that the hemolytic streptococci can be differentiated serologically by means of the precipitin reaction into distinct and sharply defined groups which are not disclosed by the agglutination reaction. She used heat and hydrochloric acid extracts of the microorganisms and the sera of rabbits immunized with formalinized cultures.

MATERIALS AND METHODS

The moths belonging to the family Phalaenidae (Noctuidae)* were obtained from the Department of Entomology, Montana Agricultural Experiment Station, and were collected in the station light traps in various parts of Montana. The specimens used were checked to genus and species by Dr. R. E. Wall of the Department of Entomology. The species used were:

Euxoa albipennis Grt.

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*Lepidoptera

Euxoa guadridentata G.&R.

Euxoa tristicula Morr.

Feltia venerabilis arida Ckll.

Feltia ducens Wlk.

Feltia vancouverensis Grt.

Sidemia devastator Brace

Agrotis ypsilon Rott.

Agrotis orthogonia Morr.

Spaelotis havilae Grt.

Protagrotis niveivenosa Grt.

Smerinthus cerisyi Kirby

Vanessa cardui L.

Scotogramma trifolii Rott.

Eumichtis loda Stkr.

Hypocoena rufastrigata Pack.

Autographa brassicae Riley

Autographa californica Speyer.

Heliothis obsoleta Fabr.

Chorizagrotis auxiliaris Grt.

The specimens which had not been held long enough to become dry were dried in an oven with the temperature at 57° C. or lower. Only the thoraces of the moths were used in the experiments because of the fact that the greatest development of muscles occurs in this body division. The legs and wings were removed to avoid the chitinous material of which

they chiefly consist. The abdomen was removed to avoid the possibility of interference of fats, oils, and intestinal contents with the reactions. The head was removed in order to avoid the possibility of the presence of an organ specific substance which might interfere with the reaction of the species specific substance.

In order to remove the appendages from the thorax, each moth was held with the thorax between the thumb and fore-finger and rolled in such a manner that the appendages were broken off. Rolling in front of an electric fan greatly facilitated the work.

Experiment with Physiological Saline Extract

The thoraces were ground up as fine as possible with a mortar and pestle. The ground material was extracted with physiological saline for two days in the refrigerator at a temperature of 3° to 6° C. before each innoculation. The details of the rabbit immunization are given in Table I.

The rabbits were bled aseptically from the heart six days after the last innoculation. The serum was separated into sterile tubes and placed in the refrigerator.

TABLE I

RABBIT IMMUNIZATION

Phalaenidae	Rabbit	Number of innoculation and amount of inoculum					
		1	2	3	4	5	6
Euxoa albipennis	1	Extract of 300 mg. in 5 cc. saline	Extract of 300 mg. in 5 cc. saline	Extract of 300 mg. in 5 cc. saline	Re-extracts from previous 3 sediments in 5 cc. saline	500 mg. in 7 cc. of saline	500 mg. in 8 cc. of saline
Euxoa quadridentata	2						
Euxoa tristicula	3*						
Feltia venerabilis arida	4						
Feltia ducens	5						
Feltia vancouverensis	6						
Sidemia devastator	7						

*Rabbit No. 3 died after the first innoculation.

Physiological saline (.85% NaCl) extracts of the 11 specimens listed in Table II were made by placing 100 mg. of the ground thoraces and 2 cc. of saline in weighed centrifuge tubes. These were kept in the refrigerator for 24 hours after which they were centrifuged and as much as possible of the supernatant liquid pipetted into labelled tubes. The residues were thoroughly dried and weighed to determine the loss in weight which was considered as the weight of the material extracted. The dilutions were made from these weights with corrections for the weight of the sodium chloride.

Tubes in which the reactions were read were made from glass tubing having an inside diameter of 4 mm. and a length of 6 cm. Approximately 0.15 cc. of serum was run into the bottom of a tube and the same amount

of diluted extract run in above the serum, care being taken to obtain a precise line of contact between the two fluids.

Because of the fact that the sera and extract dilutions were kept in the refrigerator, the reactions were read at the end of the first and second hours after being set up.

EXPERIMENTAL DATA

The results of the precipitin reactions are given in Tables II, III, IV, and V. In each table, the serum against which the extracts of the species listed were run is designated as the antiserum.

In recording the reactions, a profuse precipitate was read as four plus, a distinct ring was read as three plus, and the lighter rings as two and one plus. A reaction in which a definite ring was not formed but in which a slight precipitate was perceptable was recorded as a plus-minus reaction.

TABLE II

PRECIPITIN REACTIONS

Sub-family	Species	Undiluted	Dilution of Extract 1-10	1-20	1-40	1-80	1-100	1-160	1-200	1-500
Agrotinae (antiserum)	Feltia ducens		*	++++ ++++	++++		++++ ++++		- +	- -
Heliothinae	Heliothis obsoleta			++++ ++++	++++ ++++	+++ ++++	+ ++	- ++	- -	
Agrotinae	Euxoa quadridentata			++++ ++++	++++ ++++	± +++	- ±	- ±		
Agrotinae	Feltia venerabilis arida						++++ ++++		- +	- -
Hadeninae	Scotogramma trifolii			++++ ++++	++++ ++++	- +	- ±			
Cucullinae	Eumichtis loda			++++ ++++	++++ ++++	- +	- -	- -		
Acronyctinae	Sidemia devastator			+++ ++++	± +++	- -				
Acronyctinae	Hypocoena rufastrigata			++++ ++++	++++ ++++	- ±	- -	- -		
Plusiinae	Autographa brassicae	 -	++++ ++++	- +++	- ±	- -				
Family Sphingidae	Smerinthus cerisyi Kirby	 +++	- +++	- -						
Family Nymphalidae	Vanessa cardui L.	 -	- -	- -						

*Signs in upper part of space are readings for 1st hr., lower part for 2nd. hr.

TABLE III

PRECIPITIN REACTIONS

Sub-family		Species	Dilution of Extract 1 - 1,000 1st. hr.	1 - 1,000 2nd.hr.	1 - 5,000 1st.hr.	1 - 5,000 2nd.hr.	1 - 10,000 1st.hr.	1 - 10,000 2nd.hr.
Agrotinae	Antiserum 1	Euxoa albipennis	++++	++++	+-	++	+-	+-
	2	Euxoa quadridentata	++++	++++	+-	+-	-	-
	3	Euxoa tristicula	++++	++++		++	+-	++
	4	Feltia venerabilis arida	++++	++++		+-	-	-
	5	Feltia ducens	++++	++++	+-	+-	-	-
	6	Feltia vancouverensis	++++	++++	+-	+-	-	-
Acronyctinae	7	Sidemia devastator	++++	++++	-	-	-	-
Agrotinae	8	Agrotis ypsilon	++++	++++	-	+	+-	+-
	9	Agrotis orthogonia	++++	++++	++	++	+-	+-
	10	Spaelotis havilae	++++	++++	-	+-	-	-
Acronyctinae	11	Protagrotis niveivenosa	++++	++++		-	-	-

TABLE IV

PRECIPITIN REACTIONS

	Species	Dilution of Extract 1 - 10,000		1 - 11,000			Dilution of Extract 1 - 1,000		1 - 5,000		1 - 10,000	
		1st.hr.	2nd.hr.	1st.hr.	2nd.hr.		1st.hr.	2nd.hr.	1st.hr.	2nd.hr.	1st.hr.	2nd.hr.
1	Euxoa albipennis	-	-	-	-	1	++++	++++	++	++	-	-
Anti-serum 2	Euxoa quadridentata	++	++++	-	-	2	++++	++++	++	++	-	-
3	Euxoa tristicula	++	++	-	-	3	++++	++++	++	++	+-	++
4	Feltia venerabilis arida	-	-	-	-	Anti-serum 4	++++	++++	++	++	+-	++
5	Feltia ducens	-	-	-	-	5	++++	++++	++	++	+-	+-
6	Feltia vancouverensis	-	+-	-	-	6	++++	++++	++	++	-	-
7	Sidemia devastator	-	-	-	-	7	++++	++++	++	++	-	-
8	Agrotis ypsilon	-	-	-	-	8	++++	++++	++	++	-	-
9	Agrotis orthogonia	++	++	-	-	9	++++	++++	++	++	++	++
10	Spaelotis havilae		-	-	-	10	++++	++++	++	++	-	-
11	Protagrotis niveivenosa		-	-	-	11	++++	++++	++	++	-	-

TABLE IV (Continued)

PRECIPITIN REACTIONS

		Species	Dilution of Extract			
			1 - 5,000		1 - 10,000	
			1st.hr.	2nd.hr.	1st.hr.	2nd. hr.
Anti-serum	1	Euxoa albipennis	++++	++++	-	-
	2	Euxoa quadridentata	++++	++++	-	+-
	3	Euxoa tristicula	++++	++++	-	-
	4	Feltia venerabilis arida	++++	++++	-	-
	5	Feltia ducens	++++	++++	-	++
	6	Feltia vancouverensis	++++	++++	-	-
	7	Sidemia devastator	++++	++++	-	-
	8	Agrotis ypsilon	++++	++++	-	-
	9	Agrotis orthogonia	++++	++++	-	++
	10	Spaelotis havilae	++++	++++	-	-
	11	Protagrotis niveivenosa	++++	++++	-	-

TABLE V.

PRECIPITIN REACTIONS

	Species	Dilution of Extract 1 - 5,000 1st.hr.	2nd.hr.	1 - 10,000 1st.hr.	2nd.hr.		Dilution of Extract 1 - 5,000 1st.hr.	2nd.hr.	1 - 10,000 1st.hr.	2nd.hr.	1 - 11,000 1st.hr.	2nd.hr.
1	Euxoa albipennis	-	++	-	-	1	++	++	+-	+-	-	-
2	Euxoa quadridentata	-	+-	-	-	2	++	++	+-	+-	-	-
3	Euxoa tristicula	+-	++	+-	+-	3	++	++	++	+++	-	+-
4	Feltia venerabilis arida	-	+-	-	-	4	++	++	++	++	-	-
5	Feltia ducens	-	+-	-	-	5	++	++	++	++	-	-
Antiserum 6	Feltia vancouverensis	++	++	+-	++	6	++	++	++	++	-	-
7	Sidemia devastator	+-	+-	-	+-	Antiserum 7	+++	+++	++	++	-	+-
8	Agrotis ypsilon	+-	++	-	-	8	++	++	++	++	-	-
9	Agrotis orthogonia	++	++	++	++	9	++	+++	++	+++	+-	++
10	Spaelotis havilae	-	+-	-	-	10	++	++	+-	++	+-	+-
11	Protagrotis niveivenosa	-	-	-	-	11	++	++	-	-	-	-

The reactions recorded in Table II are the results of reactions of extracts run against the serum from the rabbit immunized against the extract of Feltia ducens of the family Phalaenidae. The negative reactions of a sphinx moth, Smerinthus cerisyi of the family Sphingidae, and of a butterfly, Vanessa cardui of the family Nymphalidae, in dilutions of 1-20 or above, point to a definite serological difference between the families.

The positive reactions of Feltia ducens and Feltia venerabilis arida in the dilution of 1-200 while the representatives of the other families showed no positive reactions in the dilution, point to a serological difference between the sub-families in the family Phalaenidae. The extract of Euxoa quadridentata gave a plus-minus reaction in the dilution of 1-160 which shows its close relationship to the other two genera of this family. The fact that Heliothis obsoleta, which is placed in the sub-family Heliothinae by Barnes and McDunnough (3) gave a two-plus reaction in 1-160, points to the fact that this species and possibly the sub-family Heliothinae are closely related to the species studied of the sub-family Agrotinae. Droudt in Seitz (19) says that although in his work he has separated this small, well defined group into the sub-family Heliothinae, its right place is in the sub-family Agrotinae. H. obsoleta is placed in the sub-family Agrotinae by Hampson (7) and also by Barnes and McDunnough (3), but McDunnough excludes it from Agrotinae in his revision of the Agrotid moths (17).

The reactions in Table III were run against the serum of the rabbit immunized against the extract of E. albipennis which is placed in the sub-family Agrotinae. The extract of E. albipennis gave a two-plus reaction against its antiserum in the dilution of 1-5000. All of the other species studied which have been placed in this sub-family by Barnes and McDunnough (3) also gave reactions in this dilution. S. devastator and Protagrotis niveivenosa which are placed in the sub-family Acronyctinae did not react in this dilution. P. niveivenosa, which was placed in the sub-family Agrotinae by both Barnes and McDunnough (3) and by Droudt in Seitz (19), did not react above 1-1000. This lack of reaction would indicate that the change from the sub-family Agrotinae to the sub-family Acronyctinae is a correct one.

Protagrotis niveivenosa which was placed in the sub-family Agrotinae by Barnes and McDunnough (3) and is now placed in the sub-family Acronyctinae by McDunnough (18) did not react above 1-1000 against the serum of E. albipennis which is placed in the sub-family Agrotinae (Table III). McDunnough (18) says that the genus Protagrotis falls, on genetalia, near Sidemia. Its reaction was similar to that of S. devastator against the same serum, but against the serum of S. devastator (Table V), P. niveivenosa did not reaction above 1-5000. This raises the question as to whether or not it belongs to either sub-family.

The extract of Agrotis orthogonia was positive in the highest dilutions against the antisera of F. venerabilis arida, F. ducens, and

S. devastator. It gave a slightly positive reaction with the antiserum of E. albipennis. The extract of E. tristicula gave positive reactions in the highest dilutions against the antisera of E. albipennis, F. venerabilis arida, E. quadridentata, and slightly positive reactions with the antisera of F. vancouverensis, and S. devastator. The inconsistancy of the results from these two extracts was at first thought possibly due to inaccurate weighing or diluting of the extracts. However, these points were checked, and the same reactions re-occurred.

Table VI gives the results of reactions of extracts run against sera obtained from rabbits immunized against extracts of A. californica, E. albipennis, and Chorizagrotis auxiliaris.

E. albipennis, which is placed in the sub-family Agrotinae, gave a four-plus reaction in the highest dilution against the antiserum of A. californica which according to Barnes and McDunnough (3) is placed in the sub-family Plusiinae. This extract also was the only one reacting against its own antiserum in the dilution of 1-20,000. More-over, against the antiserum of Chorizagrotis auxiliaris it was the only extract reacting in the dilution of 1-20,000.

These reactions do not tend to follow the differentiation obtained in the previous reactions. Hydrogen ion concentration determinations were run on all five extracts by means of a potentiometer to determine whether or not this factor was causing a discrepancy in the reactions. The determinations are given in Table VII.

TABLE VI

TITRATION OF SERA

	1-1,000		1-5,000		1-10,000		Saline	
	1st.hr.	2nd.hr.	1st.hr.	2nd.hr.	1st.hr.	2nd.hr.	1st.hr.	Checks
Autographa californica 1	++++		++++				-	-
Euxoa albipennis 2	++++		++++		++++		-	-
Chorizagrotis auxiliaris 3	++++		++++		++++		-	-

PRECIPITIN REACTIONS

Sub-family			1-5,000		1-10,000		1-20,000		1-40,000	
			1st. hr.	2nd. hr.	1st. hr.	2nd. hr.	1st. hr.	2nd. hr.	1st. hr.	2nd. hr.
Plusiinae	Antiserum A. californica	1	++++		+++	++++	+	+++	-	-
Agrotinae	E. albipennis	2	++++		+++	+++++	+	++	-	-
Agrotinae	Ch. auxiliaris	3	++++		++	+++	-	-	-	-
Acronyctinae	Sidemia devastator	4	++++		-	-			-	-
Agrotinae	Euxoa tristicula	5	++++		-	-			-	-

	1-10,000		1-15,000		1-20,000		1-40,000	
	1st.hr.	2nd.hr.	1st.hr.	2nd.hr.	1st.hr.	2nd.hr.	1st.hr.	2nd.hr.
1.	-	-	-		-		-	-
Antiserum 2.	++++	++++	+++	++++	++	++	-	-
3.	+	+	-	-	-	-	-	-
4.	-	-	-	-	-	-	-	-
5.	+	+	-	-	-	-	-	-

	1-10,000		1-15000		1-20,000		1-40,000	
	1st.hr.	2nd.hr.	1st.hr.	2nd.hr.	1st.hr.	2nd.hr.	1st.hr.	2nd.hr.
1.	-	-	-	-	-	-	-	-
2.	++++		++++		+++	++++	+	+
Antiserum 3.	;++	++	++	++	+	++	-	-
4.	++	++	-	-	-	-	-	-
5.	++	++	-	-	-	-	-	-

TABLE VII

HYDROGEN ION DETERMINATIONS ON EXTRACTS
(POTENTIOMETRIC)

Extracts	Millivolts	pH
Autographa californica 1	54.5	4.93
Euxoa albipennis 2	56	4.93
Chorizagrotis auxiliaris 3	57	4.94
Sidemia devastator 4	57	4.94
Euxoa tristicula 5	56.5	4.93

Note: The extracts were allowed to reach room temperature before these readings were made.

Extracts	Millivolts	pH
Autographa californica 1	53	4.88
Euxoa albipennis 2	56	4.93
Chorizagrotis auxiliaris 3	57	4.94
Sidemia devastator 4	57	4.94
Euxoa tristicula 5	57	4.94

Note: These readings were taken after the extracts had remained at room temperature for two hours.

Since the greatest difference between the hydrogen ion concentrations is only 0.06 it is unlikely that this factor caused the variation in reactions. Due to the lack of material it was not possible to run nitrogen determinations on these diluted extracts according to the method outlined by Dajani (4). It is very probable that such determinations would explain the differences in reaction.

Experiment with alcoholic insoluble portion of extract

In an experiment to obtain the specific immune body, 10 gr. of crushed thoraces of F. ducens was extracted for two days with 1 liter of distilled water. At the end of the first day the soaked material was reground to break up as many of the cells as possible. At the end of the second day the suspension was centrifuged and 2½ volumes of 95 per cent ethyl alcohol added to the supernatant liquid. This mixture was allowed to stand in the refrigerator for two days after which the precipitate was recovered by centrifugation. The precipitate was dried in a vacuum dessicator over sulphuric acid and dilutions made by weighing out a known amount of the dried material.

This material was dissolved in physiological saline and run against the antiserum of F. venerabilis arida and gave definite reactions in dilutions as high as 1-5,000,000.

In an attempt to show the presence of glucose in this alcoholic precipitated material, 15 gr. of ground thoraces of miscellaneous Phalaenids was extracted with 1 liter of physiological saline solution for 36 hours at a temperature of 3° to 6° C. The suspension was centri-

fuged, 2½ liters of 95 per cent ethyl alcohol added, and the mixture placed in the refrigerator (3° to 6° C.) for 24 hours after which the precipitate was recovered by centrifuging. The total yield was .450 gr. after drying. Three hundred and fifty milligrams of the dried, ground material was hydrolyzed with 35 cc. of 3 per cent H_2SO_4 for 24 hours at 100° C. The proteins were then precipitated with sodium tungstate. The resulting clear liquid was placed in a polaroscope and was found to cause no refraction of light. Twenty one and nine-tenths cubic centimeters of the material caused no reduction when run against 3 cc. of a quantitative Benedict's solution of which 25 cc. was reduced by 44.15 mgm. of glucose when standardized.

In a second experiment crushed thoraces of A. californica were treated as above and reactions run with dilutions of the material against the sera of E. albipennis, Ch. auxiliaris, and S. devastator. The reactions are given in Table VIII.

TABLE VIII

PRECIPITIN REACTIONS

	1-1,000	1-5,000	1-10,000	1-20,000	1-40,000	1-80,000
Antiserum A. californica 1	++++	++++ ++++	++ ++++	++ ++++	- +++	- ±
E. albipennis 2	++++	++ ++++	++ ++++	+ +++	- -	- -
Ch. auxiliaris 3	++++	+++ ++++	++ ++++	- ++	- ±	- -
S. devastator 4	++++	+++ ++++	++++ ++++	- ++	- ±	- -

The definite reaction in the alcoholic precipitated extract of A. californica in the dilution of 1-40,000 and its slight reaction in the dilution of 1-80,000 against its own serum while it did not react in these dilutions against the other sera shows that a higher degree of differentiation may be obtained with the alcoholic precipitated extract than with the saline extract. A. californica is placed in the sub-family Plusiinae which is the fifth sub-family following the sub-family Acronyctinae by Barnes and McDunnough (3). E. albipennis, C. auxiliaris, and E. tristicula are placed in the sub-family Agrotinae and S. devastator in the sub-family Acronyctinae.

GENERAL DISCUSSION

The remarkable development of the chemistry of specific immune bodies, and the application of immunological reactions in the determination of relationships between species of various organisms has brought to the hand of the systematist a new and exact method of determining phylogenetic relationships which should in time overshadow the long used, and often questioned, morphological characters. It is of course necessary to use the results of precipitin reactions and the morphological characters together, but the reactions can be used in deciding relationships which are questionable when based solely upon some morphological characteristic.

The taxonomy of the moths and their relationships are worked out by various authors using different morphological characters (see pages 17 and 18), with the result that names of specia have been changed. Species have been transfered from one genus to another, and genera have been

changed from one sub-family to another. The serological reactions can, without doubt, be used in deciding the correctness of these changes.

The extent of variation of the precipitin reactions necessary to show definite differences between specimens or close relationships between them could best be determined after running a large number of reactions. However, the two-plus reaction of H. obsoleta (Table II) in the dilution of 1-160 against the serum of the rabbit immunized against the extract of F. ducens shows its close relationship to the sub-family Agrotinae. This relationship has been borne out by the systematists (see page 17). The reactions of all of the species placed in the sub-family Agrotinae in the dilution of 1-5,000 (Table III) to the exclusion of the reactions of the two species placed in the sub-family Acronyctinae in this dilution show that the reading of a plus-minus reaction is possible and is definitely opposable to a negative reaction. The four-plus reaction of E. quadridentata in the dilution of 1-10,000 against the homologous serum is an excellent reading against the two-plus and plus-minus reactions of the other species which reacted in this dilution (Table IV).

SUMMARY AND CONCLUSIONS

1. Serological studies (precipitin reaction) were made of 14 genera and 20 species of moths placed in 6 sub-families of the family Phalaenidae. Reactions were also run using specimens from the families Sphingidae and Nymphalidae.

2. A specific immune body was isolated from collections of F. ducens which gave definite reaction in dilutions as high as 1-5,000,000.

3. The specific immune substance which was isolated was not hydrolyzable to reducing sugars as shown by its failure to affect polarized light or to reduce quantitative Benedict's solution after treatment with H_2SO_4 at 100° C. for 24 hours.

4. Explanations of the reactions are presented along with the placement of the various species according to a number of authorities.

5. It is evident from the reactions of the above mentioned specimens that the precipitin reaction may be used in determining a phylogenetic relationship between genera and sub-families of the family Phalaenidae.

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