

# THE RELATIONS AMONG DROSOPHILA SPECIES, AS DETERMINED BY THE COMPLEMENT FIXATION REACTION USING ETHER- INSOLUBLE FRACTIONS

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## INTRODUCTION

Serological methods have been utilized in the investigation of a great variety of animal and plant products. The especial merits of these relatively new technics have been demonstrated time and again through the solution of numerous biological problems of practical or theoretical importance. Within the last few years, several workers have applied these methods to the study of taxonomic relationships among plants and animals; and although the results of these studies have not always met with acceptance among the systematists, nevertheless, as an aid in contributing to the solution of some of the rather confusing puzzles of organic evolution, their value has come to be generally recognized.

Notwithstanding the approval that has been granted the serological approach to the study of taxonomy and phylogeny, there has been but little attention directed to the possibility of using these methods in analyzing the intricate patterns of insect relationships. Probably the explanation for the neglect that this field of investigation has suffered lies in the fact that entomologists seldom have had extensive training in the details of immunological procedure; likewise, the immunologist seldom is an entomologist as well. Whatever the cause may be, the result is unfortunate, because those few studies that have been undertaken in insect serology have met with some measure of success. Thus the work of Brown and Hefron ('28), although not extensive, suggested the possibilities

of serological reactions in distinguishing species of Lepidoptera. Another study of this group was made by Martin and Cotner ('34), and their results showed conclusively that the precipitin test could be a valuable aid in the analysis of genera and subfamilies of the family Phalaenidae. In both of these investigations the results coincided with expectations insofar as the morphological relationships were concerned. Russian investigators have used the complement fixation test to detect the presence of the Y-chromosome in males and attached-X females of *Drosophila melanogaster* (Levit, Ginsburg, Kalinin and Feinberg, '36). This, unquestionably, is one of the most delicate pieces of work attempted in this field and, if it is pursued, holds considerable promise. Finally, in this laboratory the relationships of several *Drosophila* species have been studied with both the complement fixation (Cumley and Haberman, '38) and the precipitation technics (Haberman and Cumley, in press). This report is an extension of these studies, using the complement fixation reaction with antigens prepared somewhat differently from those used in the previous works.

In the earlier investigations, antigens of *Drosophila* species were prepared by extracting the dried powdered flies with saline. No attempt was made to remove the lipoids. Antigens were standardized in terms of their nitrogen contents. In the present study those fractions of the powdered flies which are soluble in ether have been removed, and the remaining ether-insoluble powder dissolved in saline. Antigens so prepared were tested with the complement fixation reaction. The results were not found to be entirely coincident with those obtained by the previous investigations. This fact does not invalidate the results obtained by either this or the previous methods, because the materials tested were not the same. The value of any of these studies will become apparent only when a considerable mass of data, relating to several of the soluble fractions of the fly, has been assembled.

## MATERIAL AND METHODS

The powdered fly material was prepared in the following way: Flies which were grown in half-pint milk bottles on yeast-banana agar were removed from the food within 2 days after hatching. They were macerated in a mortar and then desiccated in vacuo over sulfuric acid for 2 days, at room temperature. The material was then removed, ground still more, and returned to the desiccator for further drying. When it was thoroughly dry, the powder was removed and stored in air-tight jars in the ice box.

The injection antigen was prepared by extracting the dried fly powder with 0.85% NaCl, in the ratio of 1 gm. fly powder to 10 cc. of the saline, at about 9°C. for 2 days. The broth was centrifuged and the supernatant liquid collected, filtered through several thicknesses of filter paper, and preserved with Merthiolate solution (1:10,000) in the ice box. In an earlier study data were presented regarding the water content of the flies and the solubility of the dried fly material in saline (Cumley and Haberman).

The antigens for the complement fixation tests were prepared by removing the ether-soluble fractions of the dried fly powder and then making a saline extract of the residue. This procedure was as follows: The powdered fly material was first extracted three times with ether, in the following way: 10 cc. of ether was added to each gram of fly powder; the mixture was shaken well and frequently for 15 minutes, and then filtered. To the residue the same quantity of ether was added, and the mixture was shaken, at frequent intervals, for 1 hour. The mixture was filtered again, and the residue was placed in the 40°C. incubator until no odor of ether was observed. In table 1 the data are presented regarding the extraction.

After extraction with ether, the dried powder was dissolved in saline. Twenty cubic centimeters of 0.85% saline were added to each gram of the dried powder. The mixture was allowed to remain in the ice box at 9°C. for 48 hours. It was then centrifuged; the supernatant liquid was decanted and

filtered; and the residue was dried and weighed. Table 2 presents the data regarding the saline extraction and the preparation of the antigen extracts. The nitrogen contents of these antigen extracts were determined by micro-Kjeldahl tests; the antigens were adjusted to contain equal amounts of nitrogen. In this experiment as in the earlier ones the test

TABLE 1

*Data regarding ether-extraction of fly powders*

DROSOPHILA SPECIES	WEIGHT OF DRY POWDER BEFORE ETHER-EXTRACTION	WEIGHT OF DRY POWDER AFTER ETHER-EXTRACTION	PER CENT OF ETHER-EXTRACTABLE MATERIAL IN POWDER
	<i>gm.</i>	<i>gm.</i>	
biplectinata	5.64	4.69	16.85
caribbea	4.32	3.60	16.70
funebria	4.19	2.87	31.55
hydei	4.12	3.63	11.90
melanica	7.19	6.09	15.30
melanogaster	6.67	5.69	14.70
mulleri	4.54	3.11	31.50
pseudoobscura A	1.30	1.02	21.50
repleta	1.02	0.70	31.40
simulans	3.87	3.37	12.90
sulcata	3.10	1.83	41.00
virilis	4.30	3.23	24.90

TABLE 2

*Data regarding preparation of the test antigens*

DROSOPHILA SPECIES	AMOUNT DRIED POWDER AFTER ETHER-EXTRACTION	AMOUNT SALINE ADDED TO THE POWDER	AMOUNT EXTRACT REMOVED	AMOUNT DISSOLVED POWDER IN EXTRACT	DILUTION OF THE ANTIGEN EXTRACT
	<i>gm.</i>	<i>cc.</i>	<i>cc.</i>	<i>gm.</i>	<i>gm./cc.</i>
biplectinata	4.69	93.80	81.00	2.440	1: 35.1
caribbea	3.60	72.00	57.00	1.585	1: 36.0
hydei	3.63	72.60	61.00	1.710	1: 35.6
melanica	6.09	121.80	100.00	2.685	1: 37.3
melanogaster	5.69	113.80	92.00	2.740	1: 33.5
mulleri	3.11	62.20	55.00	1.855	1: 29.6
pseudoobscura A	1.02	20.40	16.20	0.530	1: 30.6
simulans	3.37	67.40	55.00	1.480	1: 37.1
sulcata	1.83	36.60	34.00	0.605	1: 56.2
virilis	3.23	64.60	52.00	1.140	1: 45.6

antigens always have been adjusted to be of equivalent nitrogen content. This has been largely a precautionary measure, because other authors, for example, Link and Wilcox ('33), working with fungi, have demonstrated that adjustment for nitrogen equivalence had no bearing upon the antigen-antibody reaction. However, since no better method of antigen standardization was apparent, and since several investigators have considered it a desirable practice, the adjustment for equal nitrogen was employed. Table 3 is a presentation of the data regarding the nitrogen contents of the antigen extracts, before adjustment for nitrogen equivalence. These

TABLE 3

*Quantities of nitrogen in saline extracts of dried flies, previously extracted with ether, after 48 hours at 9°C.*

<i>Drosophila species</i>	<i>Milligrams of nitrogen per cubic centimeter extract</i>
bipunctinata	3.31
caribbea	2.25
funebria	1.72
hydei	2.78
melanica	2.65
melanogaster	2.65
mulleri	2.78
pseudoobscura A	3.31
repleta	2.25
simulans	2.91
sulcata	1.59
virilis	2.65

antigens were preserved with Merthiolate solution (1:10,000), and were used as the test antigens in the complement fixation reactions, the results of which are presented in this paper.

The rabbits that were used in these tests were used in the earlier experiments. The total immunization schedule included seven intravenous doses, of increasing size from 1 to 4 cc., of the injection antigen, followed by a rest interval of about a month, after which three additional injections of 2, 4 and 5 cc. of the injection solution were given to the rabbits, in order to raise their titres. Nine days after the last injection the animals were bled. The sera were collected, centrifuged, preserved with Merthiolate solution (1:10,000), and

stored in the ice box. These sera constituted the antisera used in the complement fixation reactions reported here.

The antigens were tested first for anticomplementary activity. The results of these tests are shown in table 4. As a consequence of these results, the antigens were further diluted 1:100 in order to bring the antigen dilutions well beyond the range of anticomplementary activity. The complement fixation test was then applied to each of the various antisera.

TABLE 4  
*Extent of anticomplementary activity of antigens*

TYPE OF ANTIGEN	DILUTION OF ANTIGEN							
	1: 20	1: 40	1: 80	1: 160	1: 320	1: 640	1: 1280	1: 2560
<i>D. bipectinata</i>	++++	+++	++	+	+	—	—	—
<i>D. caribbea</i>	++++	+++	+	—	—	—	—	—
<i>D. funebris</i>	++++	++++	++++	++++	++++	—	—	—
<i>D. hydei</i>	++++	++++	+++	+++	++	++	+	—
<i>D. melica</i>	++	+	—	—	—	—	—	—
<i>D. melano-gaster</i>	++	+	+	—	—	—	—	—
<i>D. mulleri</i>	++++	++++	++++	+	—	—	—	—
<i>D. pseudo-obscura A</i>	+	—	—	—	—	—	—	—
<i>D. repleta</i>	++++	++++	+++	+	—	—	—	—
<i>D. simulans</i>	++	+	—	—	—	—	—	—
<i>D. sulcata</i>	++++	++++	+	—	—	—	—	—
<i>D. virilis</i>	++++	+++	+	—	—	—	—	—

Each antiserum was diluted serially and tested against all of the antigens. Antigen dilutions were the same in all of the tubes. The results of these tests are given in table 5. Complete hemolysis in any tube was recorded as negative (—). Complete lack of hemolysis was recorded as positive (++++). Three intermediate grades of fixation were recorded as one plus (+), two plus (++), and three plus (+++), on the basis of relative amounts of hemolysis.

## OBSERVATIONS AND DISCUSSION

This investigation has brought out several facts concerning the antigenic and chemical relationships of *Drosophila* species, and it has also pointed to certain features of the technics with which other authors have computed antigen-antibody relationships among different species of animals or plants.

1. Both the Boyden ('34) and the Nelson and Birkeland ('29) methods of computing relationships have been applied to the results shown in table 5. The numerical values obtained by using these two methods are presented in table 6. Since the two technics have not yielded the same results, and since it is difficult to judge their relative merits, a brief statement will be made regarding their manipulation.

Boyden ('34) using the precipitin test, computed the percentage relationships between any two species in the following way: Assume that an antiserum reacted with its homologous antigen in highest dilution of 1:6000, whereas the same antiserum reacted with a heterologous antigen in highest dilution of 1:4000. The percentage value of relationship between the two antigens was then assumed to be  $\frac{4000}{6000}$  or 66 $\frac{2}{3}$ %. Nelson and Birkeland ('29), also using the precipitin technique, ranked wheat hybrids on the basis of the amount of reactivity, rather than the end-point of reactivity, as is characteristic of the Boyden method. The former authors assumed a (+++++) to be numerically equivalent to 10 units, and lesser degrees of precipitation were accordingly indicated. The total number of units (or +'s) was determined for the homologous antigen-antibody reaction, and this value was assumed to be 100%. Heterologous tests presented lower values, e.g., 64%, 49%, 30% and 19%, which demonstrated the degree to which the particular antigen, or species, reacted to a given antiserum. In the current investigation, both these methods of calculation were applied to the results of the complement fixation reaction. The end-point of reactivity is, in this case, the highest dilution of antiserum at which complement is fixed. The quantity of reaction was determined by counting the total number of +'s which were recorded in each

TABLE 5

*Results of complement fixation tests*

TYPE OF ANTISERUM	TYPE OF ANTIGEN	DILUTION OF ANTISERUM									
		1:10	1:20	1:30	1:40	1:50	1:60	1:80	1:100	1:120	1:160
D. mulleri (B 6) <sup>1</sup>	D. mulleri	+	+	+	+	+	+	+	+	+	—
	D. bipectinata	+	+	+	+	+	+	+	+	—	—
	D. hydei	+	+	+	+	+	+	+	—	—	—
	D. sulcata	+	+	+	+	+	+	+	—	—	—
	D. melanica	+	+	+	+	+	+	+	+	—	—
	D. virilis	+	+	+	+	+	+	+	+	—	—
	D. funebris	+	+	+	+	+	+	+	+	—	—
	D. melanogaster	+	+	+	+	+	+	+	—	—	—
	D. caribbea	+	+	+	+	+	+	+	—	—	—
	D. repleta	+	+	+	+	+	+	+	—	—	—
D. mulleri (B 5)	D. mulleri	+	+	+	+	+	+	+	+	+	—
	D. sulcata	+	+	+	+	+	+	+	+	+	—
	D. melanica	+	+	+	+	+	+	+	+	+	—
	D. melanogaster	+	+	+	+	+	+	+	+	+	—
	D. caribbea	+	+	+	+	+	+	+	+	+	—
	D. repleta	+	+	+	+	+	+	+	+	+	—
	D. similans	+	+	+	+	+	+	+	+	+	—
	D. pseudoobscura A	+	+	+	+	+	+	+	+	+	—
	D. mulleri	+	+	+	+	+	+	+	+	+	—
	D. sulcata	+	+	+	+	+	+	+	+	+	—
D. melanogaster (B 1)	D. melanogaster	+	+	+	+	+	+	+	+	+	—
	D. similans	+	+	+	+	+	+	+	+	+	—
	D. bipectinata	+	+	+	+	+	+	+	+	+	—
	D. pseudoobscura A	+	+	+	+	+	+	+	+	+	—
	D. caribbea	+	+	+	+	+	+	+	+	+	—
	D. melanica	+	+	+	+	+	+	+	+	+	—
	D. hydei	+	+	+	+	+	+	+	+	+	—
	D. funebris	+	+	+	+	+	+	+	+	+	—
	D. sulcata	+	+	+	+	+	+	+	+	+	—
	D. virilis	+	+	+	+	+	+	+	+	+	—



D. caribbea (C 4)	D. caribbea	1: 60	1: 80	1: 100	1: 120	1: 160	1: 200	1: 240	1: 320	1: 400	1: 480
	D. bipectinata	+	+	+	+	+	+	+	+	+	—
	D. hydei	+	+	+	+	+	+	+	+	—	—
	D. melanogaster	+	+	+	+	+	—	—	—	—	—
	D. melanica	+	+	+	+	+	—	—	—	—	—
	D. pseudoobscura A	+	+	+	+	—	—	—	—	—	—
	D. virilis	+	+	+	+	—	—	—	—	—	—
	D. similans	+	+	+	—	—	—	—	—	—	—
	D. funebris	+	+	+	—	—	—	—	—	—	—
	D. sulcata	+	+	+	—	—	—	—	—	—	—
	D. mulleri	+	+	—	—	—	—	—	—	—	—
	D. repleta	+	—	—	—	—	—	—	—	—	—
	D. hydei	+	+	+	+	+	+	+	+	+	+
	D. bipectinata	+	+	+	+	+	+	+	+	+	+
	D. melanogaster	+	+	+	+	+	+	+	+	+	—
	D. melanica	+	+	+	+	+	—	+	+	—	—
	D. pseudoobscura A	+	+	+	+	+	—	+	+	—	—
	D. virilis	+	+	+	+	+	—	+	+	—	—
	D. similans	+	+	+	+	+	—	—	—	—	—
	D. funebris	+	+	+	+	+	—	—	—	—	—
	D. sulcata	+	+	+	+	+	—	—	—	—	—
	D. mulleri	+	+	+	+	—	—	—	—	—	—
	D. repleta	+	+	+	—	—	—	—	—	—	—
	D. melanica	+	+	+	+	+	+	+	+	+	—
	D. sulcata	+	+	+	+	+	—	—	—	—	—
	D. caribbea	+	+	+	+	+	—	—	—	—	—
	D. funebris	+	+	+	+	+	—	—	—	—	—
	D. bipectinata	+	+	+	+	+	—	—	—	—	—
	D. hydei	+	+	+	+	+	—	—	—	—	—
	D. pseudoobscura A	+	+	+	+	+	—	—	—	—	—
	D. melanogaster	+	+	+	+	+	—	—	—	—	—
	D. mulleri	+	+	+	+	—	—	—	—	—	—
	D. similans	+	+	+	—	—	—	—	—	—	—
	D. repleta	+	+	—	—	—	—	—	—	—	—

<sup>1</sup> Numbers following type of antiserum designate the rabbit used in the experiment.

TABLE 6

*Percentage calculations from data of table 5*

TYPE OF ANTISERUM	TYPE OF ANTIGEN	PERCENTAGE ANTIGEN-ANTISERUM RELATIONSHIP: HIGHEST DILUTION OF REACTION (BOYDEN)	PERCENTAGE ANTIGEN-ANTISERUM RELATIONSHIP: TOTAL NUMBER OF PLUSSES (NELSON AND BIRKELAND)
D. melanogaster	D. melanogaster	100.0	100.0
	D. simulans	100.0	91.4
	D. bipectinata	80.0	73.9
	D. pseudoobscura A	80.0	73.9
	D. caribbea	50.0	43.5
	D. melanica	40.0	26.1
	D. hydei	30.0	13.0
	D. virilis	30.0	13.0
	D. funebris	30.0	13.0
	D. sulcata	30.0	13.0
	D. mulleri	30.0	8.7
	D. repleta	20.0	4.4
D. caribbea	D. caribbea	100.0	100.0
	D. bipectinata	55.0	63.8
	D. hydei	45.0	60.6
	D. melanogaster	24.4	37.0
	D. virilis	24.4	37.0
	D. melanica	24.4	37.0
	D. pseudoobscura A	24.4	37.0
	D. simulans	21.9	33.9
	D. funebris	21.9	30.8
	D. sulcata	18.8	26.5
	D. mulleri	13.8	17.3
	D. repleta	13.8	17.3
D. mulleri	D. mulleri	100.0	100.0
	D. bipectinata	80.0	96.0
	D. sulcata	81.7	86.4
	D. hydei	80.0	88.0
	D. melanogaster	81.7	80.4
	D. melanica	70.4	84.4
	D. virilis	80.0	76.0
	D. funebris	80.0	76.0
	D. caribbea	63.4	72.6
	D. repleta	63.4	68.7
	D. simulans	63.4	64.7
	D. pseudoobscura A	58.4	62.7
D. virilis	D. virilis	100.0	100.0
	D. melanica	50.0	55.5
	D. sulcata	37.5	51.8
	D. caribbea	37.5	51.8
	D. funebris	37.5	44.5
	D. bipectinata	37.5	40.7
	D. hydei	31.2	37.1
	D. pseudoobscura A	31.2	37.1
	D. melanogaster	25.0	29.6
	D. mulleri	25.0	2.22
	D. simulans	25.0	18.5
	D. repleta	18.8	7.4

test. By using these two different means of computation, the several species assumed essentially the same ranks of reactivity with reference to any given antiserum. This ranking on the basis of reactivity to an antiserum probably reveals the true direction of relationship of a number of species to a given species. However, these percentage relationships, as shown in table 6, are not necessarily to be interpreted as being a measure of the actual percentage of likeness and difference that exists among the various species. This actual percentage of species relationship could be determined only by using more quantitative methods, of which the technic of precipitin absorption (Duncan, '32) at the optimal antigen-antibody ratio (Dean and Webb, '26) seems at present to offer the most promising possibilities. Particularly would this be true if chemically pure antigens could be used, for Taylor, Adair and Adair ('32), using the optimal ratio technic, have been able to determine the amounts of albumin in egg white and the amounts of globulin in horse serum. Their results coincided closely with results obtained by other investigators using the ordinary technics of chemical analysis. Because of the nature of the material used in the present investigation, the accurate agglutinin-absorption method employed by Irwin and his associates (Irwin and Cole, '36 a, b; Irwin, Cole and Gordon, '36; Irwin, '38) could not be used.

2. In considering the data presented in table 6, an important fact must be borne in mind, viz., that the values indicate the ranks that the various antigens have assumed in reacting to a given antiserum, and nothing more. By way of explanation, let us consider the anti-melanogaster serum in its reactions to the various antigens. Arbitrarily, we say that the anti-melanogaster serum reacts to the extent of 100% with its homologous antigen. In terms of the extent of reactivity, based upon the highest dilution at which complement is fixed, we see that *D. simulans* antigen is inseparable from the *D. melanogaster* antigen, since the former reacts to the same dilution as does the latter. Similarly, *D. bipectinata* assumes a lower rank by virtue of its reacting to a dilution only 80%

as great as that of the *D. melanogaster* antigen. In the same manner *D. caribbea* and *D. funebris* assume still lower ranks because the highest dilutions at which they react with the anti-melanogaster serum is only 50% and 30% of that attained by the homologous antigen. The point which must be emphasized is that the reactive fractions of the *D. caribbea* antigen which cause complement fixation to occur, to a dilution 50% as great as that produced by the homologous antigen, do not necessarily include, in whole or in part, those reactive fractions of the *D. funebris* antigen which gave the 30% reaction. No doubt *D. caribbea* and *D. funebris* do share, to some extent, their antigenic fractions, but there is nothing in this particular test which would give evidence of it. Furthermore, as may be observed in table 6, antigens of the *Drosophila* species: *hydei*, *virilis*, *funebris*, *sulcata*, and *mulleri* give the same highest dilution of reactivity to the anti-melanogaster serum. This does not imply that exactly the same antigenic fractions are present in all of these species, but rather that the sum total of their various fractions which are reactive to the anti-melanogaster serum is about the same for all of the species in question. The recent work of Irwin ('38) on the *Columbidae* is relevant in this regard. He demonstrated, by agglutinin absorptions, that each species possessed definite biochemical fractions which it did not share with any of the other species tested. Also, a species possessed fractions which distinguished it from another species but which in turn it shared with a third species. It is quite probable that this fact has empirical value and that the same condition exists among the *Drosophila* species that was found to be present among the *Columbidae*. This could account, to a certain extent at least, for apparent inconsistencies that may arise in comparing morphological and serological data.

3. By comparing the data of table 5 with those of table 6 in the earlier investigation using the complement fixation reaction (Cumley and Haberman) and with those of table 1 in the investigation using the precipitation reaction (Haberman and Cumley), an interesting fact is revealed. There is considerably less variability in the reactions to the sera of rabbits

immunized to the same antigen, than in the earlier experiments wherein antigens which had not been extracted with ether were used. In the earlier investigations several large rearrangements in order of antigen reactivity were observed when the sera of two rabbits, inoculated with the same antigen, were tested. If the precipitin reaction is lipoid sensitive, as several competent workers have insisted (Boyden, '36; Moritz, '34), there is certainly the possibility that the complement fixation reaction, which probably depends upon the same antigen-antibody complex (Topley and Wilson, '36, p. 166), is subject to being affected unless non-specific lipoids are removed. Although some of the specific fractions may be eliminated by ether extraction, it is evident that the more important reactive portions remain unaltered as may be pointed out from table 5. For example, antigens prepared from *D. melanogaster* and *D. simulans* react almost identically when tested against the *D. melanogaster* B1 antiserum, whereas species much less related taxonomically to *D. melanogaster* also react only in lower dilutions. *D. melanogaster* and *D. simulans* hybridize. If specific fractions had been removed in the ether extractions, it is unlikely that any such correlation of serological and physiological characteristics would have been obtained.

4. In the previous experiments there has been a lack of reciprocal relations between antisera and antigens. The immediate study lends support to the view that reciprocity is not an essential adjunct to the antigen-antibody relationship. One may observe in table 6 that the antigen-antibody relationships are never exactly reciprocal.

5. Returning to table 1, we may observe that the amount of ether-soluble material is seen to vary considerably among the different species: *D. hydei* was found to contain 11.90% ether-soluble material, whereas *D. sulcata* was found to be 41.00% ether-soluble. We are not in a position, at the present time, to state whether this wide variation in ether-soluble fractions is due to differences in the metabolic and physiological state of the separate fly species or to differences in the food supplies available to the different species at a given time. That

is to say, we know that two species, at a given period of time after hatching, attain quite different degrees of physiological development, since there are significant differences in the duration of the life cycles of the various species. These differences in development may in part account for the differences in the percentages of ether-soluble material. In this connection it is of interest to note that, in table 1, *D. simulans* and *D. melanogaster* are shown to contain 12.9% and 14.7% ether-soluble material, respectively; whereas *D. mulleri*, *D. sulcata*, *D. virilis*, and *D. repleta* contain from 24.9% to 41.0% ether-soluble constituents. *D. melanogaster* and *D. simulans* are closely related morphologically and physiologically and have approximately a 10-day life cycle, whereas the other four species have a much longer life cycle and are taxonomically quite distinct from *D. melanogaster* and *D. simulans*. Consequently, it may well be that the quantity of ether-soluble material in the fly body is a function of its physiological age, although in table 1 there are species featured which do not comply with expectations in this regard. If there were a similar correlation between physiological age and the synthesis of antigenic constituents in the organism, then there would be introduced in the present investigation a possible source of error. The correlation of morphological and antigenic development is a field which has hardly been touched. Consequently, until further study is made in this direction, we must proceed with the assumption that each of the fly species is more or less the antigenic equivalent of any of the rest. And in any event, the differences in ether-soluble fractions emphasizes the importance of ether extraction of the test antigens.

6. By ranking the species in terms of the reactivity of their antigens to a given antiserum, certain features may be noted. Species which are known to be closely related taxonomically are also closely related serologically. For example, as may be seen in table 6, *D. melanogaster* and *D. simulans*, which produce a hybrid, both possess antigens which react to the extent of 100% when tested against anti-melanogaster serum.

Species which are known to be distantly related taxonomically, to a given species, usually are indicated as being distantly related serologically, as well. Thus, in Sturtevant's ('21) systematic account of *Drosophila* species, *melanogaster* and *simulans* have been placed in a subgroup different from that in which *sulcata*, *virilis*, *repleta*, *hydei*, and *mulleri* have been placed. Antigens prepared from the latter group of species reacted to anti-*melanogaster* serum to a dilution of 20% or 30% as high as did antigens prepared from the former pair of species. Occasionally, antigens which are distantly related to a given antiserum will rearrange themselves and exhibit inconsistencies. Or sometimes an antigen, prepared from a species known to be morphologically distant in its relationship to the species used in producing the antiserum to be tested, will appear to be closely related rather than distantly related to the antiserum. Discrepancies of this sort were not frequent and could probably be attributed to a lack of refinement in the tests at the present stage of the investigation, or to error in reading the tests.

#### SUMMARY

1. Antigens used in the present experiment are shown to be less variable in their reactions than antigens which were not pre-extracted with ether. The presence of ether-soluble fractions in antigens probably leads to irregularities in results.
2. Antigen-antibody relationships were not reciprocal.
3. The serological ranking of species more or less conforms to expectations on the basis of taxonomic criteria. Discrepancies are probably due to inaccuracies in performing or reading the tests.

#### ACKNOWLEDGMENTS

The author wishes to acknowledge the helpful criticism of Profs. J. T. Patterson, T. S. Painter, and W. S. Stone of the department of zoölogy and Profs. I. M. Lewis and V. T. Schuhardt of the department of bacteriology of the University of Texas.

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