WING VEIN DEVELOPMENT IN CROSSVEINLESS-LIKE STRAINS OF DROSOPHILA MELANOGASTER[1]

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THE analysis of adult phenotypic differences resulting from experimental or genetic modification of a developing system sometimes yields a useful description of the course of development in that system. Such a description may include, explicitly or not, the ontogenies of the ultimate character differences; therefore, it is desirable that the basic information should include the morphological facts of those ontogenies. At issue here is not only the possibility that the causes of the development may be recognized in preadult structure, but as well the requirement that the structure assumed in theory must be kept consistent with the structure in fact. This paper provides part of the morphological information, in anticipation of any possible speculation, for a phenogenetic analysis of polygenic crossveinless-like (cvl) strains of *Drosophila melanogaster*.

The general information of Drosophila wing development is already provided in detail by AUERBACH (1936) and by WADDINGTON (1940). This has been summarized by BODENSTEIN (1950), whose own observations were in essential agreement. The present study is restricted to that part of the total development in which the wing veins are becoming manifest, and further limited by an emphasis upon the formation of the posterior crossvein and its associated longitudinal veins. Within these confinements we not only can confirm that part of the earlier description of normal development, but we recognize new variant patterns of wing vein formation among genetically different strains of crossveinless type. Consideration of these facts will aid in understanding of the epigenetics of Drosophila crossvein determination.

MATERIALS AND METHODS

Most of this paper is concerned with two crossveinless-like lines obtained by phenotypic selection from mass-mated wild-type strains. The wild-type sources of the selections were from original wild collections, geographically distinct for the two cvl lines. The selected lines are designated cvl-5 and cvl-6, the latter including two sublines, cvl-6a and cvl-6b, derived by parallel selections out of the same source. The original selections were for high penetrance. Following upon the first studies of pupal wing development, cvl-5 and cvl-6b were each separated by new selection into a line with high penetrance and a line with low penetrance. The cvl-6a did not respond to selection for reduced penetrance.

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A third line, provided by DR. ROGER MILKMAN and designated cve (MILKMAN 1960a, b), forms a smaller part of this study.

Controls for the normal pattern of vein development include Ore-R and wild-type lines derived from the Orinda (Ona) and Riverside (Riv) parental sources of cvl-5 and cvl-6 respectively. A comparative basis for understanding the formation of crossvein interruptions is provided by a less extensive study of the mutants *cv* (1–13.7) and *cv-c* (3–57.9).

Isogenic material isolated from the cvl and wild sources was used in some comparisons. The mutant markers and balancers were those of the *Cy/Pm; D/Sb* stock of the Department of Zoology at Berkeley (STERN). Later trials used a similar stock in which the *Cy*-marked *SM1* from Pasadena (LEWIS) and the *D*-marked *CxF* from Bloomington (MULLER) were substituted for the corresponding member of the balancer stock. A detailed description of the program of crosses is not published here but will be made available on request.

The animals supplying the wing mounts were grown in a water bath at 25°C upon the standard cornmeal-molasses-agar medium, supplemented by thick suspensions of live yeast. The individuals that had formed puparia were collected hourly and placed in a petri dish containing moist filter paper. By convention, pupal ages are timed from puparium formation, thereby including the prepupal period. In these cases the time of pupa collection is defined as time zero. The dishes containing collected pupae were incubated in cans immersed in the 25° bath until the age for fixation was reached. The pupae were fixed by pouring boiling water on them; then they were stored in 70 percent alcohol until dissected. Dissection is, in this case, simply a matter of removing the pupa from its puparium and divesting it of its wings. The pupae were hardened for a time in xylol just before dissection. This treatment seemed to make easier the removal of the cuticle from around the wing. In preliminary study, the wings were stained with aceto-orcein as a matter of convenience. As the stain does give a good picture of the developing wing veins, we continued to use it exclusively. After staining, the wings were dehydrated in 95 percent alcohol and mounted whole in Euparal. All work was done on the microslide, each slide having the two wings of a single pupa.

RESULTS

The nature of the pupal wing vein: The veins in adult wings of wild-type Drosophila are a series of interconnected tubes of thick chitin associated with the remains of densely packed cells in the wing surfaces. The story of the formation of these veins includes the process or processes of forming and maintaining spaces, corresponding in position to those of adult veins, between the two layers of the pupal wing. The formation of these spaces, except for the marginal vein (WADDINGTON 1940), precedes any other sign, such as increase in cell density, of differentiation to a definitive vein. These facts color the description following in subsequent pages, so that it is stated in terms of the apparent nature and origins of the spaces to be seen in successive stages.

The presence of a lumen in the pupal wing veins was demonstrated with sectioned material by WADDINGTON (1940). Fortunately, there are criteria by which the presence of the space can be inferred from whole mounts. For one thing, the two layers of wing cells are easily distinguished from each other. It can be verified by focusing that the vein surfaces bulge away from each other in contrast to the closeness of the surfaces in the nonvein regions. The bulge is to be expected as the effect of a space left between the two wing surfaces. A more significant detail shows up quite well in the photographs of Figures 1, 2 and 3. This is the granular appearance of the tissue in the nonvein regions. In the veins, the background stain varies in intensity, being often darker than in the rest of the wing; but it is

always much more homogeneously distributed. Thus at levels of focus between the two layers of cells the vein and nonvein regions are, as a rule, quite easily distinguished by their staining properties. According to WADDINGTON (1940), the two layers of cells in the nonvein areas are in contact by way of cytoplasmic extensions or processes that are not formed by the cells around the veins. Just as WADDINGTON interpreted it, we suppose that the heterogeneity observed in the whole mounts is a representation of the cytoplasmic processes, probably distorted by fixation damage. The homogeneous staining is evidence of the blood filled lumen of the forming vein.

As was indicated already, these qualities of the lumina of the developing veins are the qualities allowing for the recognition of the veins in the pupal wing. When the veins are considerably narrowed, the contrast between vein and nonvein becomes enhanced by the increasing density of cells over the former.

The progress of wing vein development with age: As described by WADDINGTON (1940), the pupal wing begins with the end of expansion of the prepupal wing into a bloated sac. In the pupa this sac contracts into a flat blade, and the lumina of the crossveins and of the longitudinal veins, except L2, come to be established as remnants of the preceding single, continuous space. The coming together of the two wing surfaces begins at the distal margins and at the base of the wing (see Figures 2a,b), so that the longitudinal veins first appear in broad outline in these regions (see, for example, Figures 1a,b). The uncontracted middle portion is called the "central vesicle." The formation of cellular processes, extending between the two cell layers in nonvein areas, progressively blocks off the central vesicle into the middle portions of L3 and L4, the distal part of L5 and the posterior crossvein (Figure 1c). Once set apart the veins narrow to adult proportions by continuing formation of cellular processes along their margins (Figure 1d). The last vestige of the central vesicle disappears as the posterior crossvein narrows in proportion to the longitudinal veins (Figures 1e,f).

Within any one age class of a single genotype there is often some variation in the level of vein development. In order to make comparisons between strains, we have developed a system of descriptive grades defined, separately for longitudinal veins and the posterior crossvein, in terms of the degree to which the veins have narrowed. A detailed description of these grades is given in Table 1, which also points out the matching wings of Figures 1 and 2. Formulated independently of WADDINGTON's (1940) description, these successive grades correspond to the details of progress of development from the end of his stage P2b through the first half of stage P2c. The formation of the anterior crossvein, which comes about with the ultimate narrowing of L3 and L4, does not vary among the strains studied here; therefore no attempt to include it is made.

A sample of developmental grades within an age class can be conveniently summarized by its arithmetic mean. Similarity of the wings of a single fly appears more frequently than is expected of coincidence; therefore the fly, not its wings, is treated as the random variable and the average values range from 0 to 6 for the longitudinal veins and from 0 to 4 for the crossvein. (In Table 2 the maximum for the longitudinal veins is 4, as the grades 1 and 2 were not distinguished in

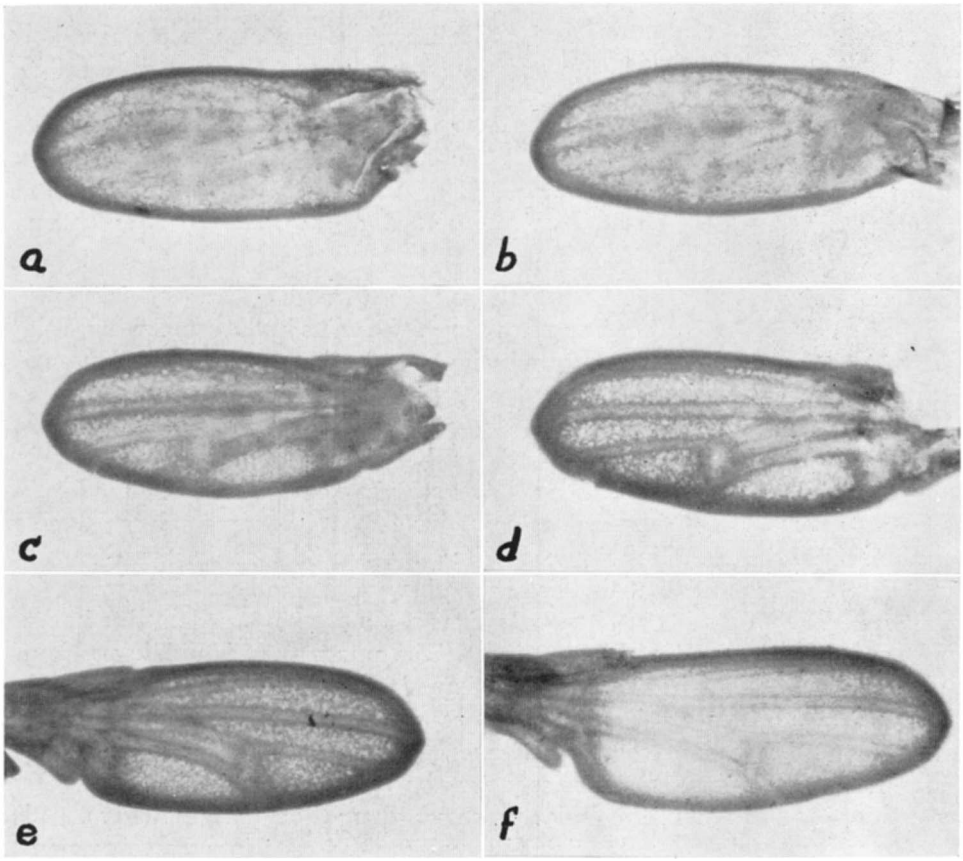

FIGURE 1.—Pupal wings of Ona wild type: a—21 hr; b—23 hr; c—24 hr; d—26 hr; e—28 hr; f—30 hr.

the earlier work.) The differences between grades cannot be treated quantitatively as linear step increases because the actual developmental equality of any two steps has not been demonstrated. Nevertheless, within any strain the temporal sequence of average grades does correspond to the temporal sequence of qualitative levels of development. It can be expected, therefore, that gross differences between strains in the progress of the wing veins will be reflected in differences in the calculated averages.

Most of the crossveinless-like lines are like wild type in the timing of vein formation. In Table 2 are listed the selected cvl lines that were included in the preliminary study. Most entries are based on a sample of approximately ten animals; some few for wild and for cvl-6b are from about 20 flies. No differences appeared among several wild-type sources that were examined; these are pooled in the table as "wild type." In this case the veins begin to form at 21 hours and reach the maximum recognized by our scheme before 27 hours. A small sample of pupae of *cv-c*, not included in the table, fit very well to this same sequence of

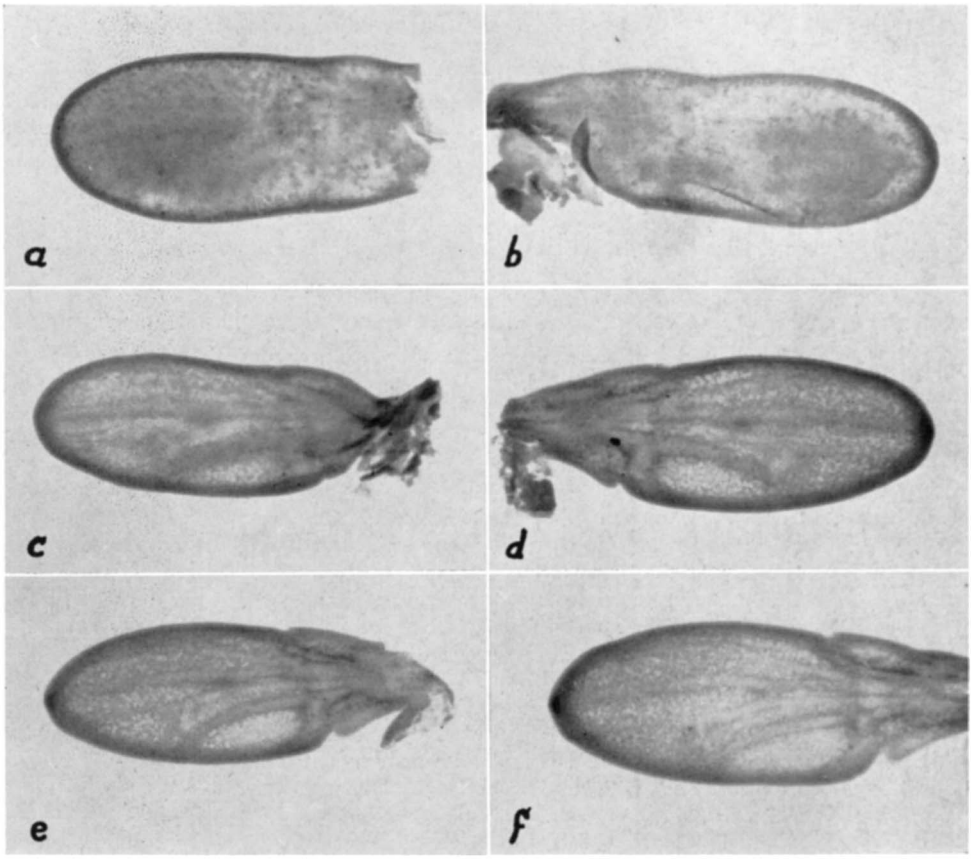

FIGURE 2.—Pupal wings of cvl-5: a—22 hr; b—24 hr; c—26 hr; d—28 hr; e—30 hr; f—32 hr.

timing. MILKMAN's cve is indistinguishable from wild type in the timing of vein formation. With respect to the longitudinal veins, the cvl-6 lines are also like wild type in timing. The crossveins have been left off here because the scheme describing crossvein development is not applicable to the cvl-6 flies. The nature of the cvl-6 crossvein is discussed in a subsequent section.

Though the scheme of describing vein development is appropriate for cvl-5, the timing is different from that of wild type or any of the other lines. The rate within the period when changes are occurring is not different, but the initiation and the completion of the sequence is delayed 4 to 5 hours. Comparison of Figures 1 and 2 can illustrate the similarity of later ages in cvl-5 (Figure 2) with early ages of wild type (Figure 1). For example, Figure 1c, the wing from a 24-hour wild-type pupa, can be matched with Figure 2d from a 28-hour cvl-5 pupa. Similar matching of later and of earlier wings can be made between the two plates.

The delay seen with cvl-5 is not a matter of generalized slowing down of the developmental rate over the entire life of the fly. Such a decrease, equal to 4 hours

TABLE 1

The stages of vein formation in pupal Drosophila

Grade	Description	Figures where illustrated
A.	*Stages of longitudinal vein development.*	
0	Only the very most distal part of the wing manifests differentiation of longitudinal veins. This grade is always associated with grade 0 of crossvein.	2a,b
1	L3 and L4 are both outlined in the distal region. L5 is separated from L4 proximally to central vesicle.	1a,b and 2c
2	All longitudinal veins are present in distinct outline distally, but still only broadly indicated toward the base. Vein L2 is not fully developed.	1c and 2d
3	All veins have narrowed to an approximation of the adult proportions.	1d,e,f and 2e,f
B.	*Stages of posterior crossvein development.*	
0	Either nothing present as in grade 0 of the longitudinal veins or there may be at most a very large central vesicle with grade 1 longitudinal veins; see Figure 1a as an example of the latter.	1a and 2a,b
1	The posterior crossvein is still represented by a central vesicle which is reduced in relative size and more distinctly bounded than is the grade 0 central vesicle. This occurs with grades 1, 2 and 3 of the longitudinal veins.	1b,c,d and 2c,d
2	The posterior crossvein is narrowed to limits that are in proportion with the longitudinal vein development. This occurs only with grade 3 of longitudinal veins.	1e,f and 2e,f

TABLE 2

The average developmental grade of wing vein by pupal age

		Age in hours												
Strain	Vein type*	21	22	23	24	25	26	27	28	29	30	31	32	33
"wild"	LV	0.8	1.8	3.2	4.0	3.8	4.0	4.0	4.0	..	4.0	..	..	..
	CV	0.2	1.6	2.0	2.0	3.0	3.6	4.0	4.0	..	4.0	..	..	..
cve	LV	0.5	1.7	2.4	4.0	4.0	4.0	4.0	4.0	4.0	..	..	..	..
	CV	0.1	0.5	1.2	2.0	3.6	4.0	4.0	4.0	4.0	..	..	..	..
cvl-5	LV	..	0	0	0	0.2	0.7	1.8	3.2	3.2	3.6	4.0	4.0	4.0
	CV	..	0	0	0	0.2	0.6	1.3	1.8	2.1	2.4	4.0	3.4	4.0
cvl-6a	LV	1.3	2.0	3.2	3.3	3.9	4.0	4.0	4.0	4.0	..	..	..	..
	CV	..	..	..	..	..	..	..	..	..	..	..	..	..
cvl-6b	LV	0.5	1.5	3.3	3.7	4.0	4.0	4.0	..	4.0	..	..	..	..
	CV	..	..	..	..	..	..	..	..	..	..	..	..	..

* LV is the mean grade of longitudinal vein development; CV is the corresponding mean for crossveins.

in 24 or 1/6, would delay puparium formation almost a whole day and eclosion at 25°C would be retarded by 1½ days. A general slowdown operating only during pupal life would work a 16-hour delay on eclosion. Cultures from eggs collected at hourly intervals were examined on the fifth day for puparium formation. Part of these were reexamined on the ninth day and another part on the tenth day for the proportion of flies emerging as adults. Each culture was counted for the number of pupae present at only one time interval and for the number eclosed 96 or 120 hours later. In Table 3 each entry is the pool of several independent cultures. Under *n* is the number of pupae forming in the cultures or the total number of flies finally emerging. The second number is the percent that had formed pupae or emerged by the end of the time interval indicated. It is seen that cvl-5 is no slower to begin puparium formation than is wild-type Ona. Nor is there any difference in time of eclosion. Therefore, the cvl-5 delay is limited to some time between puparium formation and the first appearance of vein changes. We have not looked at any correlated developmental changes, so there is no positive information to distinguish between a delay of all systems and a delay specific for the wing veins.

Formation of interrupted posterior crossveins: In agreement with WADDINGTON's (1939) description of the *cv* mutant, there are no visible crossveins in the 24-hour pupae of the mutants *cv* and *cv-c*, just as in the adult. (The small piece of vein of adult *cv-c* is not noticed in the pupal wing.) At 21 hours the central vesicle does form in the fashion described for wild type, but it fails to persist to form a crossvein. While the longitudinal veins are still in grade 2 (Table 1A) in 22–23 hour old pupae, the two layers of the wing come together, obliterating the space of the crossvein; cellular pocesses form; and development proceeds as it does in any nonvein region.

In the wings of adult cvl flies a variable portion of the posterior crossvein in missing. In the pupal wings of cvl flies there are seen clearcut discontinuities,

TABLE 3

Times of puparium formation and eclosion of Ona and of cvl-5

Strain	Experiment I						Experiment II			
A. Puparium formation										
	Time interval in fifth day						Time interval in fifth day			
	112–116 hours		118–122 hours		124–128 hours		114–116 hours		118–122 hours	
	n	%	n	%	n	%	n	%	n	%
Ona wild	44	32	28	71	34	85	134	17	204	46
cvl-5	129	25	117	74	40	80	190	34	301	67
B. Eclosion										
	Time interval in tenth day						Time interval in ninth day			
	232–236 hours		238–242 hours		244–248 hours		210–212 hours		214–218 hours	
	n	%	n	%	n	%	n	%	n	%
Ona wild	43	98	28	97	32	100	129	16	197	33
cvl-5	120	96	111	97	39	100	184	11	283	43

which we call "interruptions," separating the crossvein into two parts or setting it apart from one of the longitudinal veins. Examples are apparent along L5 in Figures 2e,f. The interruptions do not occur in wild type; and, as shown below, their frequency corresponds to the cvl penetrance in the wings of adult flies. These are, therefore, the pupal cvl phenotype. For both cve and cvl-5 the formation of the interruptions seems to be like the formation of the *cv* and *cv-c* phenotypes. Though total obliteration of the space of the central vesicle rarely occurs in these cvl strains, the interruptions appear in the way and at the time relative to longitudinal vein development outlined for the mutants. As illustrated for cvl-5 by Figure 2c, the central vesicle of cve and of cvl-5 is still complete in the early stages with grade 1 (Table 1A) longitudinal veins. Subsequently the layers of the wing come together in part of the central vesicle, closing off the space of that portion. Presumably the remainder of the central vesicle forms the fragment of crossvein that appears in the adult. Of course, in cvl-5, where the vein development is delayed, the appearance of the interruption is correspondingly tardy.

The formation of posterior crossvein interruptions in cvl-6 is different. Complete interruptions occur already with the first signs of longitudinal veins at 21 and 22 hours. L4 and L5 are entirely separated, leaving no central vesicle. As the longitudinal veins narrow, the space between them widens, still containing no fragment of crossvein. Figures 3a,b are examples of these stages. Yet by 24 to 25 hours there are posterior crossveins present in the majority of the wings. Closer examination of the intervening period reveals what we refer to as a secondary formation of the posterior crossvein.

At 22 and 23 hours in that region normally containing the posterior crossvein, there appear irregular open spaces we call "gaps." The gaps lack the heterogeneity of the nonvein region and lack the homogeneous staining of the central vesicle. These unstained spaces, arrows in Figures 3c and d, are not holes in the wings; in the original whole mounts both layers of cells can be seen with the gap between them. The appearance is that of a space devoid of cytoplasmic strands and of blood. In other slides of flies in the 23 to 24 hour period the crossvein cannot be classed unambiguously as typical crossvein or as "gap" crossvein; there is an increased density of cells, but the margins are less clearly bounded than are those of normal pupal crossveins. These are interpreted as representing a stage intermediate between the gap and the typical crossvein. Figures 3e,f are photographs of two "intermediate" wings from 24-hour old pupae. We believe that the gap arises by withdrawal of the cell processes formed with the early closure of the central vesicle. Around the new space a crossvein is formed.

If the new categories of crossvein are successive states, we should expect a shift in relative frequencies with age. About 100 wings were mounted for each of the age intervals at 22, 23, 24, 25 and 26 hours and classified as typical (perhaps interrupted but otherwise indistinguishable from wild type of a comparable age) or atypical (showing no crossvein or appearing as either of the new classes of crossvein). For the typical group the numbers showing early (grades 0 or 1 of Table 1B) or terminal (grade 2 of Table 1B) stages are distinguished; for the atypical group each of the three possible classes are counted. Figure 4 illustrates

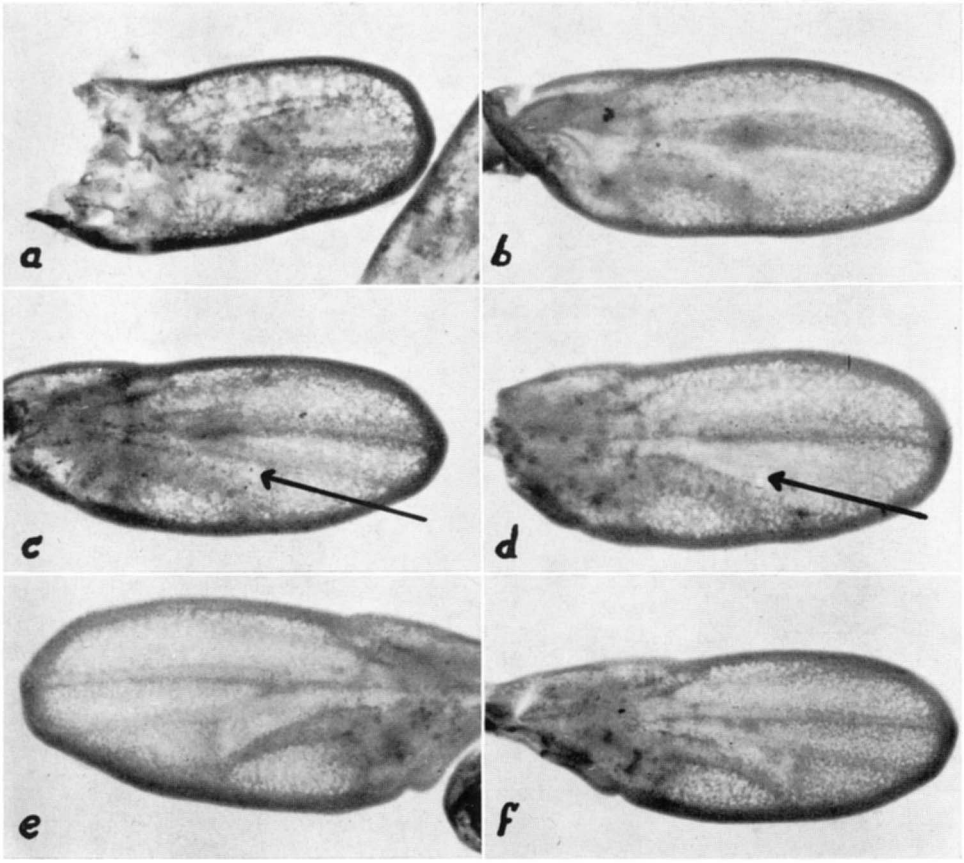

FIGURE 3.—Pupal wings of cvl-6b: a, b—21 and 22 hr, respectively, early interruption; c, d—23 hr "gap" crossvein; e, f—24 and 25 hr, respectively, "intermediate" crossvein.

the results. On the bottom line is revealed a striking difference between the number of initial central vesicles at 22 hours and the number of apparently typical terminal crossveins at later hours. The upper line displays the proportions of the total wings with an atypical aspect to the crossvein region. The conversion of the crossveinless class first to the gap stage, then to the intermediate state is inferred from the shift in relative frequencies with time. Since the total of atypical crossveins declines, the intermediate class is not a terminal state. There is no decrease in recovery of examinable wings so the intermediate class is not disappearing into death or technical oblivion. Rather, the secondary sequence of changes leads to a crossvein structure indistinguishable from the typical posterior crossvein.

Thus for cve and cvl-5, as for the mutants *cv* and *cv-c*, the central vesicle comes first. This space is closed down later to produce absence of the crossvein. In cve and cvl-5 a part of the original space remains to produce a crossvein fragment in the adult. For cvl-6 the central vesicle is rarely found; rather, the space around which the adult crossvein fragment is organized appears secondarily. That is, the

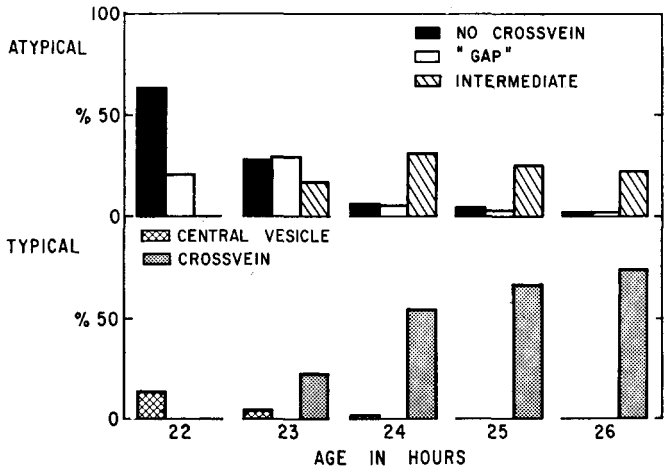

FIGURE 4.—Classification of cvl-6b pupal crossvein with age. The crossveins are distinguished as "typical" (lower) and "atypical" (upper) as explained in the text. Within each line successive stages in the formation of crossvein are defined and their relative proportions indicated by the bar heights. The ordinate for each row is the percent of the total age sample.

"gap" of cvl-6 does not have continuity with the space formed by prepupal expansion.

The frequency of crossveinless-like phenotypes in pupal wing: The simplest picture of these two sequences of crossvein development and interruption would imagine the cvl-5 phenotype to be the end product of a partial destruction of crossvein. Conversely, the cvl-6 phenotype would be conceived as the end product of the incomplete construction of crossvein. Pictures of this kind would predict a period of change in crossveinlessness with time, so that cvl-5 becomes visibly more interrupted and cvl-6 becomes visibly less so. Alternatively, the actual proportion becoming interrupted might depend on factors not to be deduced from these pictures.

For cvl-5 the earliest wings show no interruptions. A few interruptions are already apparent in crossvein grade 1 (Table 1B) at 26 or 27 hours. At 28 hours, 5 of 16 wings at this level show an interruption; by 29 and 30 hours, 22 of the 31 wings of grade 1 are interrupted. For more advanced wings, crossvein grade 2, Table 4A demonstrates that there is no further increase (or decrease) in interruption frequency over the level achieved by 29 or 30 hours. It is probable that there is also no change in quantity of interruption per wing after this time; the proportion of interrupted wings that lack crossvein entirely stays constant after 30 hours when first recorded.

The wings of cvl-6 start with no crossvein. The conditions such as "gap" are so irregular in shape that any distinction between interrupted and complete crossvein is impossible to make. Consequently, the counts are necessarily limited to the final typical stage in the crossvein formation. As shown on Table 4B, at all ages after 24 hours, the frequency of interruptions has achieved the level of

TABLE 4

Frequency of interruptions among pupal wings in late stages of crossvein formation

A. Late wings* cvl-5					B. Late wings* cvl-6				
Pupal age (hr)	Total wings	Interrupted number	percent		Pupal age (hr)	Total wings	Interrupted number	percent	
28	1	0			23	31	10	32	
29	3	2	60		24	86	36	42	
30	4	3			25	52	19	37	
31	20	14			26	66	27	41	
32	20	15			28	137	57	41	
33	20	12			35	73	33	45	
34	20	14	64		Adult ♀♀	226	123	54	49
35	21	12			♂♂	184	77	44	
36	20	12							
37	14	9							
Adult ♀♀	216	157	72	67					
♂♂	262	163	62						

* "Late wings" are those in which the crossveins are classed in grade 2 (Table 1B).

the adult unweighted mean and does not change, even though the proportion of typical crossveins does increase over part of this time span (Figure 4). Thus the interruption frequency at the final stage of crossvein formation is independent of the age at which that level is reached. It remains possible either that in the course of forming the secondary crossvein the frequency of interruption declines with developmental stage to this determined level, or that at its beginning the secondary development includes just this much of the crossvein, no less and no more.

Whatever does occur to the interruption frequency, it is of value to notice that in the pupal wings it is determined by 24 hours in cvl-6 or by a corresponding 29 hours in cvl-5. The genetics allows for considerable variation in penetrance. These penetrance controls are likely to operate, then, in advance of the critical stages recognized here.

The genetics of the crossveinless-like strains: By way of marked balancer techniques, the major chromosomes of cvl-5 and of cvl-6 have been isolated in non-cvl backgrounds. In both cases the studies were first made with the earlier lines. Following separation of these lines into high and low penetrance sublines, the major chromosomes were reisolated from all sources and programs constructing combinations of isogenic chromosomes from different sources were begun. The notation for any isogenic stock mentions only the chromosomes from sources other than the multiply marked balancer stock. That is, "cvl-5 II" has the second chromosome from the original selection, but the X (unmarked) and the third chromosomes (*D/Sb*) come from the marker stock. Similarly, "cvl-5 III" has the X and the second chromosomes (*Cy/Pm*) from the marker stock. The new combinations are also named by the *origins, not the phenotypic effect*, for all major chromosomes; for example, Riv X; cvl-5hi II; cvl-5hi III, alternatively Riv X; cvl-5hi A,

has the X-chromosome taken from the Riverside wild type and the two major autosomes isolated from the cvl-5hi selection.

MILKMAN (1960a, b) has shown that cve differs from wild type by a polygenic system distributed over all of the major chromosomes. The phenotypes of the chromosomal isolates from cvl-5 and from cvl-6 (either 6a or 6b) demonstrate that in each line a gene or genes of a single chromosome are a major necessary factor for the departure from normal crossvein phenotype. In the case of cvl-5, these genes are on the third chromosome; for cvl-6 the major gene has been localized on the X-chromosome to a single locus (not *cv*). Genetic localization of cvl-5 is incomplete. For both cvl-5 and cvl-6 the penetrance of the cvl phenotype and its correlated expression varies with various substitutions in the remaining major chromosomes. The genetic differences of this latter kind are called "modifiers". The differences between high and low penetrance lines depend on such modifier differences. The details of this analysis are being prepared for publication elsewhere (MOHLER, 1965).

Since the genetic differences between the selected lines and wild type are several, we wish to know which, if any, of these are responsible for the departures from normal in the pupal wings. So far our studies recommend that the phenotype of development and the phenotype of the adult crossvein both depend upon the same major gene or genes. Variation in the modifier genes, however, has not been associated with an unambiguously correlated variation in development.

Genetics of cvl-5 retardation: Just as the phenotype of cvl-5 depends on a major gene or genes in the third chromosome, so does the delay in vein development depend upon the third chromosome. The result of a preliminary study of second and third chromosomes isolated from the original cvl-5 selection is given in Table 5. The hours 22, 26 and 30 were chosen as significant points of difference between cvl-5 and the wild type of Table 2. These are reentered in Table 5 for comparison. It is seen that the strains with the cvl-5 third chromosome are slower in initiating development than are those with a non-cvl chromosome III. In

TABLE 5

Mean stage of longitudinal vein (LV) and of crossvein (CV) in flies with isolated cvl-5 autosomes

		Age of pupae in hours		
		22	26	30
Ona (wild type)	LV	3.7	6.0	6.0
	CV	1.8	3.8	4.0
cvl-5 II (*D/Sb*)*	LV	2.5	6.0	6.0
	CV	0.8	4.0	4.0
cvl-5 III (*Cy/Pm*)*	LV	0.2	3.7	6.0
	CV	0	2.0	4.0
cvl-5 (selection)	LV	0.1	2.2	5.8
	CV	0	0.7	3.8

Each entry is the average of 9 or 10 pupae.
* Isogenic stocks as explained in the text.

Table 5 there is evidence that the second chromosome from cvl-5, which increases penetrance, also acts to retard the beginning of vein development—compare Ona with cvl-5 II at 22 hours, and cvl-5 III with cvl-5 at 26 hours. The combination of the delays shown by the isolated second and third chromosome stocks does account for the total delay of cvl-5. It might be supposed that these delays in development are directly related to the causes of the adult cvl phenoype. However, the isogenic material from the second selection series will not support this conclusion.

In the first place, the isogenic X-chromosomes also modify penetrance and expression. The order of their effects is cvl-5hi X > Riv X > Ona X > cvl-5lo X. Yet these X-chromosomal differences are not correlated with differences in developmental timing. In Table 6, Riv X and cvl-5hi X are compared in cvl-5hi A background; Ona X, cvl-5hi X and cvl-5lo X are matched in Ona A background. It is seen that there is no variation in timing with variation in X chromosome.

A more critical contradiction is seen in the delays that do occur. The major delay of four hours as an effect of the third chromosome is confirmed—compare the timing of Ona A stocks with the limited data from Ona X; Ona II; cvl-5hi III in Table 6. But the second chromosome effecting higher penetrance, i.e., cvl-5hi II, *shortens* the delay to 2 to 3 hours rather than lengthening it—compare the timing of Ona A stocks with the cvl-5hi A stocks.

The genetics of cvl-6 secondary crossvein: The overall rate of vein formation in cvl-6 is, from Table 2, quite like that of wild type. The difference is in the qualitative features of the formation of the crossvein. In each of the three different isogenic cvl-6bhi X lines, the autosomes of which come from non-cvl-6 sources, the pupal wing veins are those characteristic for cvl-6. Since the X-chromosome is also the site of the cvl-6 major gene, it is likely that this major gene, and cer-

TABLE 6

Mean longitudinal vein (LV) and crossvein (CV) stages in pupae from isogenic stocks of cvl-5 and wild type

Isogenic stock		Age of pupae in hours							
		20	22	24	26	28	30	32	34
Riv X; cvl-5hi A	LV	0	1.0	1.8	*3.2*	*4.6*	*5.8*	6.0	6.0
	CV	0	0	0.1	*2.0*	*2.2*	*3.3*	3.8	4.0
cvl-5hi X; cvl-5hi A	LV	0.4	1.3	2.2	*3.8*	*5.2*	*6.0*	6.0	6.0
	CV	0	0	0.2	*1.8*	*2.2*	*2.6*	4.0	4.0
Ona X; Ona A	LV	0	0.7	*3.6*	*5.7*	6.0	6.0	6.0	..
	CV	0	0	*1.9*	*3.0*	3.3	4.0	4.0	..
cvl-5hi X; Ona A	LV	0.6	2.2	*4.4*	*6.0*	6.0	6.0	..	6.0
	CV	0	0.5	*2.1*	*2.7*	3.9	4.0	..	4.0
cvl-5lo X; Ona A	LV	0	1.1	*4.7*	*5.2*	6.0	6.0	6.0	6.0
	CV	0	0	*2.0*	*2.2*	3.3	4.0	4.0	4.0
Ona X; Ona II; cvl-5hi III	LV	.	0	0.6	2.3	*3.4*	..	..	..
	CV	.	0	0	0.3	*2.0*	..	..	..

Each entry is the average of ten pupae except for Ona X; Ona II; cvl 1-5hi III, in which only six to eight animals are used for each number.

tainly not an autosomal gene, is responsible for the departure from normal development.

Whatever it is that modifying genes do to alter the adult penetrance in cvl-6 lines, they do not appear to alter the course or rate of crossvein formation. Table 7 includes two low penetrance lines and two high penetrance lines derived from cvl-6 sources. The frequency of the adult phenotype is given in percent from samples of approximately 100 of each sex. The development is presented as samples of wings taken one at random from each of 25 flies and classified according to the description developed in the earlier section. Two conclusions are apparent: (1) The proportion of wings with atypical early development is the same regardless of penetrance. Thus the complete crossvein of low penetrance lines and the interrupted crossvein of high penetrance lines are formed by the same secondary sequence. (2) The rate of progress through the secondary sequence is not consistently related to the ultimate penetrance shown by the isogenic lines.

To be sure, cvl-6blo X; cvl-6bhi A does seem to proceed more rapidly than the rest. It is possible that the autosomes of cvl-6bhi contribute to higher penetrance by mechanisms related to a faster rate or earlier beginning of crossvein formation and that other modifiers, as on the X chromosome, do not function this way. On the other hand, in the cases of two combinations of cvl-6bhi X with cvl-5hi III development begins *tardily* but follows the course typical of cvl-6. Penetrance is 100 percent in such combinations and expression is more extreme than for isolated cvl-6bhi X or cvl-5hi III (MOHLER, unpublished).

DISCUSSION

The formation of crossveinlessness proceeds from the elimination of the lumen of the central vesicle around which a posterior crossvein normally is to be organized. In all cases, the formation of an interruption requires that the wing surfaces at the crossvein join together as they do in any nonvein region. Contrary to experience with some of the crossveinless mutants, the developmental patterns of cvl-5 and cvl-6 differ from the wild type in ways other than the obvious failure

TABLE 7

Penetrance and crossvein development in isogenic lines of cvl-6

Isogenic line	Penetrance (percent cvl) ♀♀	♂♂	Pupal age* 22 hours typ	no	gap	int	23 hours typ	no	gap	int	24 hours typ	no	gap	int
cvl-6ahi X; cvl-6blo A	12	2 (low)	3	17	3	2	0	8	14	3	0	3	14	8
cvl-6bhi X; cvl-6blo A	73	31 (high)	0	14	11	0	0	7	15	3	1	3	14	7
cvl-6blo X; cvl-6blo A	10	8 (low)	2	12	7	4	0	6	19	0	2	0	15	8
cvl-6blo X; cvl-6bhi A	94	82 (high)	2	14	5	4	0	7	9	9	2	3	9	11

* Entered in each column is the number of pupae of that class in a sample of 25 as explained in the text. typ=typical crossvein; no=crossvein absent; gap="gap" crossvein; int=intermediate crossvein.

of the crossvein. For cvl-5 the difference is in a shift of an otherwise normal pattern of vein development to a later time; the formation of interruptions occurs during the later stages of longitudinal vein development, much as it does for *cv*, *cv-c* and cve. For cvl-6 the events of vein formation are occurring at a normal time, but the pattern of crossvein formation is markedly altered as a consequence of an early obliteration of the central vesicle.

Each of these different modifications of vein development is identifiable with the chromosome responsible for the switch from normal adult phenotype. It may be hypothesized that both the change in pupal phenotype and the adult phenotype depend on the same major gene or genes in each case. In contrast, nothing has been observed in the pupal development that explains the modifying effects of other chromosomes.

In the case of cvl-5 the information on the effects of modifier chromosomes may even contradict the hypothesis that the delay of development is relevant to the occurrence of crossvein defects. The fact is that the isogenic line Ona X; Ona II; cvl-5hi III is slower than cvl-5hi X; cvl-5hi A to initiate vein development, yet it is of lower penetrance than is the cvl-5hi isogenic stock. Thus if the pupal delay and the adult cvl are pleiotropic effects of the same genes, they certainly cannot be ordered in a cause-effect relationship. On the other hand, the methods used so far cannot distinguish pleiotropism from linkage. The cvl-5 major gene, if any, must be more rigorously identified before the delay of vein formation can be demonstrated to share its genetic cause with that of the cvl phenotype.

It is easier to associate the cvl-6 pupal phenotype with crossvein interruption since the forerunner of the normal posterior crossvein is removed early in the course of events. The final crossvein results from what has been termed "the secondary sequence". The conditions allowing the secondary sequence are probably not a property of cvl-6 strains alone. These lines establish a unique, *early* interrupted condition from which the secondary sequence is derived. Our interpretation of the new pattern requires the recognition that there is in the normal fly some property inhibiting the formation of cellular processes in the region of the posterior crossvein. In the normal fly, this property must come into operation at least by the time the central vesicle is becoming progressively narrowed. Early interruptions as in cvl-6 may not encounter this effect, so that cellular processes are formed. When the inhibitory condition appears in this case, the effect is to withdraw the cellular processes once formed in the region of the posterior crossvein. Since the central vesicle itself is not present in cvl-6, the hypothetical property is not of the contents of the forming veins but is of the wing cells regionally specialized to resist the formation of connections between the wing layers. This must be a "wild type" property so that it is unlikely that the cvl-6 major gene should produce a defect on adult wings as long as this property remains intact. Yet other genes influencing the quantity or quality of this property could affect the likelihood of the cvl phenotype. These genes would be "modifiers". The modifiers would have no necessary effect upon the course or upon the rate of the secondary sequence; they could affect only the amount. So far that is consistent with the absence of developmental effect of the cvl-6 modifiers.

Thus "gene action" for cvl phenotype of cvl-5 and of cvl-6 is not explained by their respective departures from normal development. In the first case, the effects may be irrelevant; in the second case the pattern is derivative. The "gene action" in each case lies in some *unknown* damage to the crossvein system. However, our observations do give some clue as to the nature of the damage. In each case, the effect of the cvl genes must be on some part of the mechanisms that normally operate to establish or maintain a lumen between the wing surfaces. Furthermore, we can expect that the barriers to forming interruptions must be different at succeeding stages of development. The fact that the interruptions occur differently relative to the state of longitudinal vein development means that cvl-5 III and cvl-6 X do different kinds of damage.

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SUMMARY

Three lines, each with similar crossveinless-like phenotypes, were studied for the pupal development of wing vein formation and the formation of crossvein interruptions. MILKMAN's cve appears to develop all veins in a quite normal fashion. The crossvein interruption appears by joining of the wing surfaces in part of the crossvein region subsequent to the establishment of the potential crossvein. This differs from the process seen in *cv* and *cv-c* mutants only in the degree to which the crossvein is obliterated. The cvl-5 line does not differ in the sequence of vein formation or in the manner of forming interruptions. It does, however, display a very striking delay in the initiation of the vein formation. The cvl-6 lines form longitudinal veins in a time and in the manner expected of wild type. They differ in that the wings begin vein development with a joining of the wing surfaces through the crossvein region. Partial repair is accomplished by a secondary developmental sequence including a withdrawal of the cell processes joining the wing surfaces at this position and a subsequent reorganization of a crossvein around this "gap".

Genetic studies on cvl-5 and cvl-6 reveal that the major chromosome necessarily responsible for the departure from normal adult phenotype is also largely responsible for the unique aspects of development in each. Though the cvl penetrance in both stocks is also affected by modifiers distributed over the major chromosomes, there have been no unambiguous clues to the modifying mechanisms uncovered in pupal development.

It is argued that the gross features of timing in cvl-5 development may be irrelevant to the production of crossvein interruptions. Also the striking features of the cvl-6 pattern are probably derivative and, therefore, of little help in explaining the cvl phenotype. Still the manner in which the visible interruptions first come about must be different in the two systems.

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