Temperature Effects on Day Old *Drosophila* Pupae

ROGER MILKMAN

From the Department of Zoology, Syracuse University, Syracuse

ABSTRACT Day old *Drosophila* pupae were subjected to a variety of closely controlled temperature shocks. Twenty-five hours after puparium formation (at 23°), temperatures from 39.5–41.5° (Q_1 = 2.3) differentially disturb the formation of the posterior crossvein. Three other separate treatments disturb posterior crossvein formation: treatments in the range 36.0–37.0° at 25 hours; 37.3–37.8° at 25 hours; and 39.5–41.5° at 19 hours. Certain qualitative effects are associated with certain temperatures: elliptical holes are seen in wings of flies exposed 25 hours after puparium formation to temperatures from 37.3–37.8°. Anterior crossvein defects ensue if animals are similarly exposed to temperatures from 37.9–38.2°. Within the physiological range, animals raised at higher temperatures are more resistant to the effects of temperatures at 39.5–41.5°. An extremely rapid temperature adaptation by exposures to temperatures in the range 31–38° results in markedly greater resistance to heat shock; here resistance to production of crossvein defects increases faster than to death. The association between qualitative effects and treatment temperatures is modified by changing the temperature at which the animals spend their first day of pupal life. Summation experiments support conclusions drawn from the simpler experiments. Genetic variation and interspecific variation are discussed in the present context, as well as implications of the role of protein denaturation in the biological effects of high temperatures and further, more general experiments.

INTRODUCTION

The posterior crossvein of *Drosophila melanogaster* provides a good biological end point for the study of temperature effects. The structure is linear, and its development is more labile at certain temperatures than any other contemporaneous observable developmental process. Moreover, study of this crossvein does not require its isolation, and so we can draw conclusions with some assurance as to the validity of measurements made and their importance to the organism. In a study originally designed to lay the foundation for a sensitive analysis of genetic variation, a number of rather remarkable phenomena were observed. The investigation of various temperature effects on day old *Drosophila* pupae consequently took on an interest all its own, particularly in

the light of recent contributions to the understanding of the general mechanisms of temperature effects (see, for example, references 3, 4, 11, 12). The work to be presented in this paper covers a group of experiments relating to the production and prevention, by exposure to certain temperatures, of a variety of morphological responses.

MATERIALS AND METHODS

Pupae of *Drosophila melanogaster* were collected within an hour after puparium formation (before noticeable tanning took place) and aged in vials in a water bath controlled to within 0.1°C of the desired temperature. At the appropriate time (corresponding to 25 hours at 23°C after collection) the animals were treated at a given temperature in a similar water bath controlled to within several hundredths of a degree Centigrade. After treatment the animals were placed in an incubator at 25°C and the adults emerging about 4 days later were examined.

When pupae were placed in vials, the warm-up time from 25 to 40.5°C was found to take 5 minutes by thermocouple measurements, and other evidence corroborates this finding. It was possible, however, to eliminate this warm-up period by placing the pupae in teabags, which were then immersed in the water bath for the desired treatment. The direct immersion in water did not harm the animals additionally. The teabag method was used mainly in parts of some of the rapid temperature adaptation experiments.

Posterior crossvein defects were quantified by dividing each posterior crossvein into imaginary sixths. The two posterior crossveins of the fly then totaled 12 parts. A fly was rated as to the number of twelfths that were missing irrespective of the particular location of the crossvein defect. Flies might, therefore, be rated anywhere from "0" (normal) to "12" (posterior crossveins completely absent). A fly rated "9" would have nine-twelfths of its posterior crossveins missing. Unless otherwise stated, animals used for these studies are all highly inbred "isogenic" flies of the Oregon R strain.

Methodology applicable only to a particular kind of experiment will be discussed with that experiment.

OUTLINE OF PHENOMENA TO BE DISCUSSED

Pupae treated in the manner described above in the temperature range from about 39.0 to about 42.5°C show defects of the posterior crossveins when the duration of treatment is appropriate. Within the range of 39.5 to 41.5°, the process of interference with normal crossvein formation has a Q_1 of 2.3, implying that protein denaturation is involved. At 39.0° it takes longer than expected to produce the effects. Above 41.5° death often precedes the production of crossvein defects. Detailed presentation of the dosage-response, age-response, and temperature relationships is made in a previous paper (9), but Fig. 1 summarizes the relevant findings reported in that paper. It will be seen from Figure 1 *a* that the age-response curve is bimodal; moreover, recognizable

differences between the classes of crossvein defects produced at the two different times imply two different sensitive processes.

The points in Fig. 1 *b* represent the responses to various treatment durations at various temperatures. The lines drawn through them are based on three assumptions, which they test: (*a*) Duration-response linearity. (*b*) A common point of departure (*y*-intercept) for each sex. (*c*) A temperature coefficient of 2.3 for 1°C.

The significance and validity of these assumptions and the reasoning behind them will be discussed below.

In addition to these two sensitive steps in posterior crossvein formation, two other processes (or combinations of processes) must be invoked to explain the results observed, for there are two other discrete temperature ranges at which posterior crossvein defects can be produced 25 hours (at 23°) after puparium formation.

At this very time, other temperatures produce qualitatively different effects. At 38.0°, defects of the anterior crossvein are produced. Though the posterior crossvein is undamaged, a tendency towards "leakiness" of all the veins (in which little dots and streaks of vein material are observed near the various veins) occurs, together with some slight modifications of the bristles. In the 37.5° range, doses greater than those necessary to produce posterior crossvein defects result in the wings being much smaller than normal, held out, and having a remarkable elliptical hole in their centers. Where the hole does not prevent its being seen, an unusual approximation of the third and fourth longitudinal vein is observed. Table I summarizes the responses at half-degree intervals.

All the foregoing responses pertain to flies raised at 23°. Naturally the sensitive periods would be earlier for flies raised at higher temperatures and later for flies raised at lower temperatures. It is not difficult to delineate the sensitive period appropriate to each temperature. What is striking, however, is the fact that the temperature at which the animals are raised has a profound effect on the *response* to treatment, not only quantitatively but qualitatively. As might be expected, animals raised at a higher temperature are more resistant to various treatments. Totally unexpected, however, is the association of certain qualitative responses with a given narrow temperature range which depends upon the temperature at which the animals were raised. For example, the hole in the wing which appears at 37.5°, in the case of animals raised at 23°, appears at 38.0° in the case of animals raised at 28°. Various other cases will be discussed.

Increased resistance to treatment in the 39.5 to 41.5° range (as measured by crossvein defects or by death) is conferred with remarkable rapidity by prior exposure to temperatures from 31 to 38°. As little as 10 seconds in this range will increase the minimum lethal exposure at 40.5°. Pretreatments

lasting from 45 to 60 minutes will enable the pupae to survive exposures as long as 100 minutes. Certain combinations of pretreatments will permit survival of 135 minutes. Resistance to crossvein defect production is conferred preferentially, for animals pretreated in this manner at durations of 5 minutes or longer emerge normal if they emerge at all.

A number of experimental approaches designed to throw light on some of the more technical questions encountered in the course of this investigation will also be discussed.

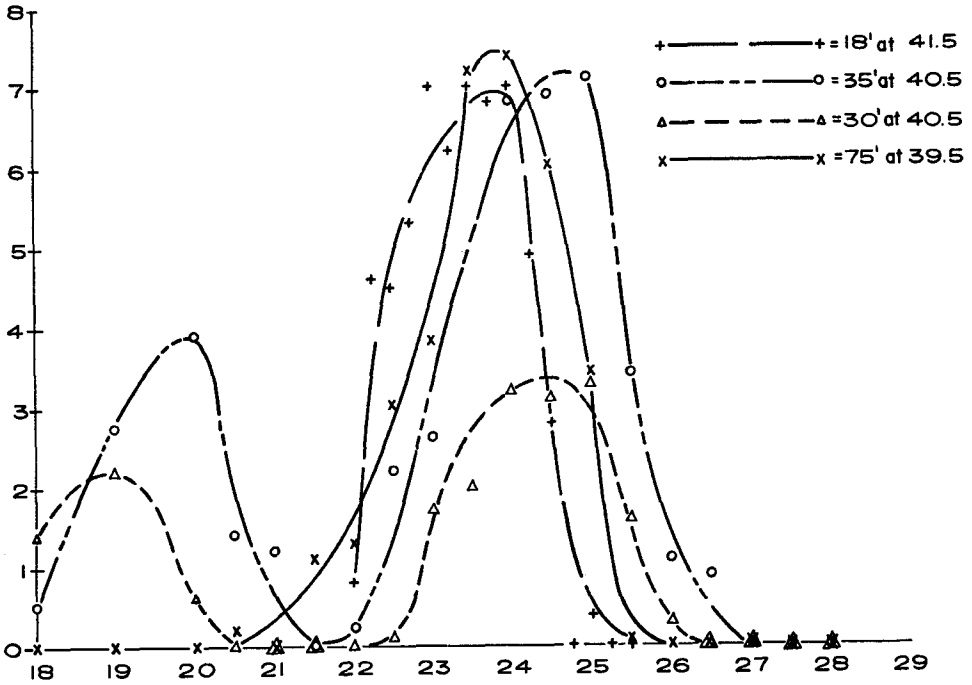

FIGURE 1 *a*. Response (12ths of posterior crossvein missing) *vs.* age (hours after pupation) at various treatments. Only two of the series were run over the whole range.

RESULTS

Posterior Crossvein Defects

A brief summary will be made of findings previously reported (9). Production of posterior crossvein defects by heat is a threshold phenomenon, and after the threshold is passed, the average response seems to rise linearly with increasing dose. The response of the two sexes is characteristically different, that of males beginning later and increasing faster than the response in females. Temperatures at 0.5° intervals between 39.5 and 41.5° (5 in all) were compared as to their effectiveness in the production of crossvein defects by comparing dura-

tions at the various temperatures which produced equivalent responses. The calculation of relative effectiveness, *i.e.* temperature coefficient, was made independent of the dosage-response relationship. It was found that a Q_1 of 2.3 fits the data quite well, and, by converting treatments at the various temperatures to equivalent times at 40.5°, all the data could be plotted with response as a linear function of time. The existence of a threshold implies that whatever is being destroyed exists in the animal in greater than necessary amounts, so that the destruction of some of it does not lead to visible results. The simplest assumption concerning the invisible destruction of this substance is that it

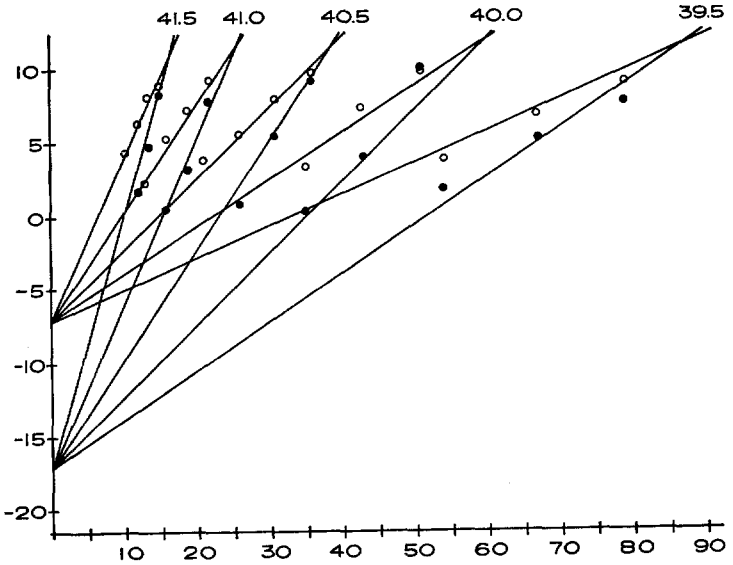

FIGURE 1 *b*. Average response (12ths of posterior crossvein missing) *vs.* duration of treatment. Open circles, females; filled circles, males. Lines drawn from intercepts with slope varying on the assumption that $Q_1 = 2.3$. Intercepts and slope at 40.5°C calculated from data pooled on the assumption that $Q_1 = 2.3$.

proceeds at the same rate above the threshold as below it. This assumption provides a basis for the extrapolation of the dosage-response line back to zero time, the time at which the animals reach bath temperature.

Because of the remarkably high temperature coefficient, corresponding to an activation energy of about 160,000 calories per mole, it is reasonable to assume that the substance being destroyed is a protein or a function of a protein. The y-intercept, where the dosage-response curve reaches zero time, is a measure of the amount of reserve protein (or protein function) in the same units as those in which the crossvein defects are mentioned. Thus -12 represents the ability in reserve to make a complete set of posterior crossveins. The intercepts have been estimated as follows: for females -7 and for males

-17. Each point in Fig. 1*b* represents between 50 and 100 animals. There is considerable variation from fly to fly but much less from vial to vial. Each vial contains 20 to 40 animals of both sexes and, of course, only half of these would appear on a given point.

To summarize these findings, we may state a formula which systematizes all the information:

$$r = kt\,2.3^{(T_t - 40.5)} - f(T_a)$$

where r = average crossvein defect rating
k = rate of destruction of crossvein-making ability for the sex in question. For males $k \cong 0.75$, for females $k \cong 0.5$

TABLE I
EFFECTS PRODUCED BY TREATMENT OF 25 HOUR PUPAE (23°C) AT VARIOUS HIGH TEMPERATURES

Temperature	Morphological effects
°C	
39.5–41.5	*cve*; $Q_1 = 2.3$
39.0	*cve*
38.5	None
38.0	"38.0 complex"
37.5	"37.5 complex"
37.0	Slight *cve*
36.5	*cve*
36.0	Slight *cve*
35.5	None

cve = posterior crossvein defects.

This constant is independent of T_a
t = dosage in minutes
T_t = treatment temperature, °C.
f is a function as yet undescribed
T_a = temperature °C, at which the animals spend their first pupal day (biological time)
$kt\,2.3^{(T_t - 40.5)}$ represents the rate of destruction (slope)
$f(T_a)$ is the reserve crossvein-making ability (y-intercept, or I)

Fig. 2 illustrates graphically the relationships involved here. Fig. 3 is a nomogram which can be used to calculate one unknown variable if one knows the other two. It is based on the same mathematical relationship but has the additional advantage of relating three variables at once.

It has been seen from Fig. 1 *a* that the sensitive period is moderately long and that it is bimodal. The response at 19 hours is separated from that at 25

hours by a period which is relatively refractory to the induction of posterior crossvein defects. One may further distinguish defects produced at the two periods from one another by the fact that an all-or-none response is frequently observed for the 19 hour period and almost never for the 25 hour period. Thus a fly with one perfect crossvein and one crossvein completely missing is characteristic of the earlier period. Kinetic studies of the production of defects in the earlier period are still in process.

In a temperature range around 37.5°, posterior crossvein defects are also produced. These can sometimes be distinguished from defects produced at

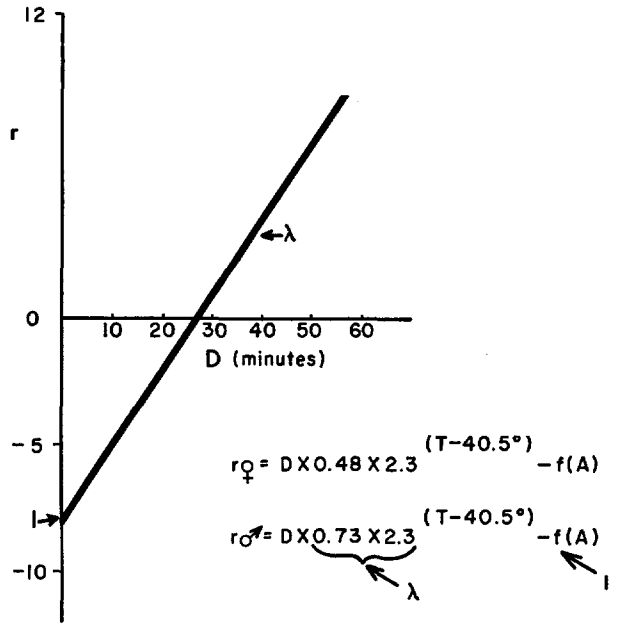

FIGURE 2. Schematic representation of destruction of crossvein-making ability with formula described in text.

other temperatures by the fact that they often begin in the middle of the posterior crossvein. In contrast defects in the other ranges almost invariably begin at the fourth longitudinal vein. Crossvein defects are also produced at about 36.5°. Fig. 4 illustrates the relative effectiveness of various temperatures in the production of posterior crossvein defects, together with the optimal treatment durations.

Qualitative Effects Associated with Certain Temperatures

Flies raised at 23° show qualitative effects associated with certain temperatures. There are two of particular interest. One observed in the range of 37.3

to 37.8° (not at 37.9° and not at 37.2°) is the presence in the wing of an elliptical hole. This hole may be produced by treatments ranging in length from 4 to 4¾ hours. It is of further interest that the position of the hole ranges continuously from proximal at the highest effective temperature to distal at the lowest effective temperatures. The longer treatments produce holes of such size, however, that they take up most of the wing. Associated with this effect

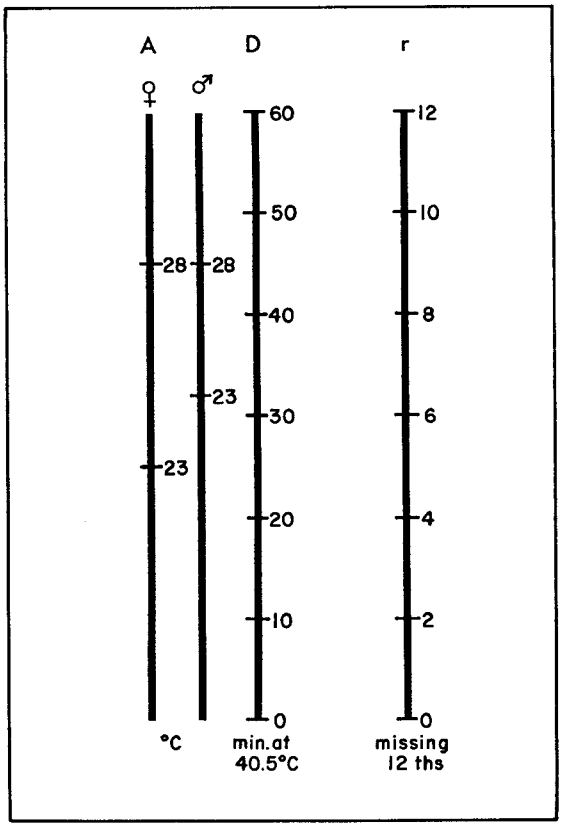

FIGURE 3. Nomogram relating temperature of first day of pupal life (*A*); treatment duration at 40.5° (*D*); and response (*r*). The *D* line can be altered to fit any temperature from 39.5 to 41.5° using $Q_1 = 2.3$.

are a number of less exotic ones. The wings are a good deal smaller than normal and are delicate-looking. They are held out from the body, and longitudinal veins 3 and 4 are abnormally close to one another. A second qualitative effect is seen in the range from 37.8 to 38.1°. This is the absence of the anterior crossvein. In this range, also, there is a certain tendency for slight bristle defects. It should be emphasized that this association of these effects with these temperature ranges holds only for flies raised at 23°.

Temperature Adaptation

Sensitivity to treatments at high temperatures is reduced in flies raised at temperatures in the physiological range higher than 23°. This is observed in terms of the amount of time needed to produce crossvein defects and to produce death. Table II relates the duration of exposure to 40.5° necessary to produce an average crossvein defect rating of 9 in flies raised for their first

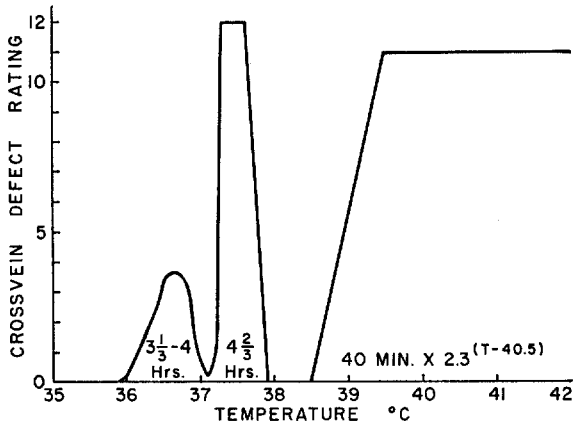

FIGURE 4. Temperatures effective in producing crossvein defects in 25 hour pupae. Optimal dosages are listed.

TABLE II

EFFECT OF TEMPERATURE AT WHICH EARLY PUPAL LIFE IS SPENT ON SENSITIVITY TO EXPOSURE TO 40.5°C

Temperature	Age of maximal sensitivity	Min. at 40.5° producing crossvein defect rating of 9
°C	*hrs.*	
18	42	30
19	—	—
20	32½	33
21	30½	32
22	27½	36
23	25	35
24	22¾	36
25	22	37
26	20½	41
27	19¼	43
28	18½	46
29	18½	48
30	18½	53
31	18½	58

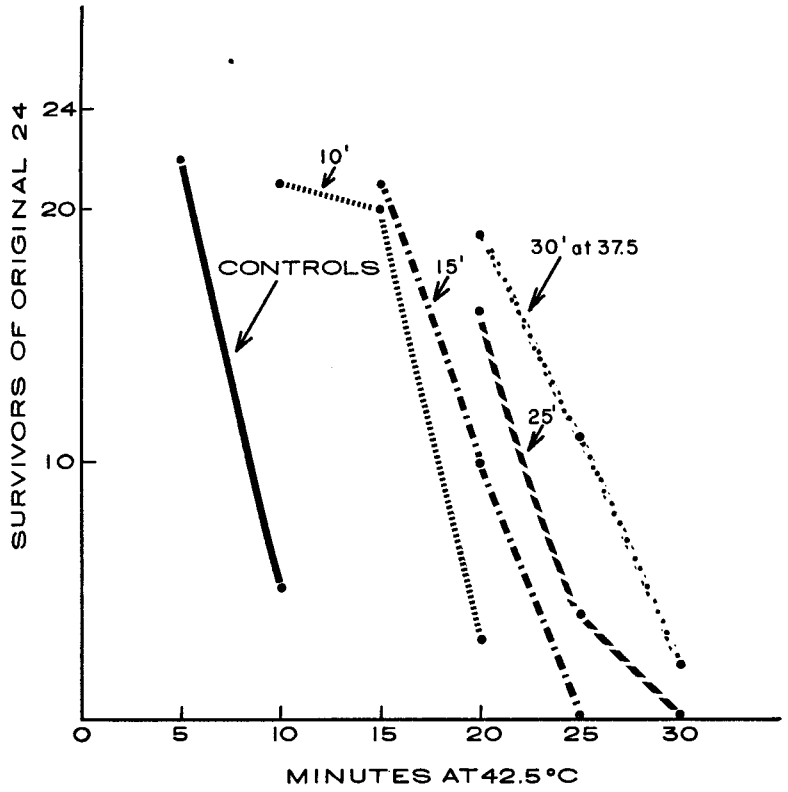

FIGURE 5. Survival curves for treatment at 42.5°C after pretreatments at 37.5° for various durations.

TABLE III

NUMBER OF ADULTS (OUT OF 30) EMERGING AFTER TREATMENT FOR 60 MINUTES AT 40.5°C, 24 HOURS AFTER PUPARIUM FORMATION

In parentheses, number of *cve* individuals

Pretreatment age, *hrs.*		22	21	20	19	18
Interval between pretreatment and treatment, *hrs.*		2	3	4	5	6
Min. at 37.5°	10	29 (0)	14 (0)	11 (4)	5 (5)	3 (3)
	5	18 (0)	5 (1)	0	0	1 (3)

LD_{100} (without pretreatment) $\cong$ 45 min.

day of pupal life (or equivalent) at various temperatures in the physiological range. It will be seen that the higher the temperature, the more effective is a difference of 1°. Exposure to these higher temperatures in the physiological range for just a few hours before the treatment at 40.5° increases resistance to the heat shock fractionally. The data in Table II may be made to fit an em-

TABLE IV

PER CENT SURVIVAL WITH VARIOUS PRETREATMENTS AND TREATMENTS

Each value represents from 12 to 60 flies treated.

		Min. at 40.5°							
		50	60	70	80	90	100	120	150
Controls		0	0	0	0	0	0	0	0
Pretreatment									
Temperature	Duration								
°C	min.								
38.5	10	—	0	—	—	0	—	0	0
	20	—	20	—	—	0	—	0	0
	30	—	10	—	—	0	—	0	0
37.5	10	80	50	10	0	0	0	—	—
	15	—	80	—	0	0	—	—	—
	20	—	75	50	0	0	0	—	—
	30	—	80	90	0	0	0	—	—
	40	—	80	80	20	—	0	—	—
	60	—	80	50	60	20	0	—	—
36.5	10	—	50	0	0	0	0	—	—
	15	—	75	10	0	0	0	—	—
	20	—	80	50	10	10	0	—	—
	25	—	75	70	20	20	—	—	—
	30	—	100	80	60	20	—	0	0
	60	—	80	—	—	50	—	0	0
36.0	10	100	90	70	30	0	—	—	—
	20	100	100	90	80	—	—	—	—
	30	100	90	90	100	—	—	—	—
35.0	10	70	50	0	10	—	—	—	—
	20	80	70	50	40	—	—	—	—
	30	90	80	90	70	—	—	—	—
34.0	10	80	50	0	—	—	—	—	—
	20	90	95	80	90	—	—	—	—
	30	90	100	50	100	—	—	—	—
33.0	10	100	30	30	5	—	—	—	—
	20	100	90	90	75	—	—	—	—
	30	90	95	75	80	—	—	—	—
32.0	10	80	100	75	10	—	—	—	—
	20	100	100	90	80	—	—	—	—
	30	100	90	90	100	—	—	—	—
	240	—	—	—	—	—	—	0	0
31.0	10	—	25	0	—	—	—	—	—
	20	—	40	—	—	—	—	—	—
30.0	10	—	10	—	—	—	—	—	—
	20	60	—	—	—	—	—	—	—

pirical formula implying that a certain proportion of the treatment is independent of raising temperature and that another part is dependent on it, but the relationship of this interpretation to reality is not clear.

A far more striking kind of temperature adaptation is seen in the prior exposure of pupae to temperatures in the range of 31 to 38°. As little as 10 seconds at 36.5° markedly increases the duration of exposure to 40.5° (or any other temperature from 39.5 to 42.5°) which the flies will survive. And, in contrast to the effects of temperatures in the physiological range, pretreatment in the 32 to 38° range produces *differential* adaptation. With pretreat-

TABLE V

REDUCTION IN RESPONSE TO TREATMENTS AT 40.5° AFTER SHORT TREATMENTS AT 37.5°

Average crossvein defect is given and numbers surviving out of 12 pupae are listed in parentheses

Controls	Min. at 40.5°C 34	40	45
None	4.5 (11)	8.4 (5)	– (0)
10 sec. at 25°			– (0)
Pretreatments at 37.5°			
10 sec.	3.2 (12)	7.3 (10)	9.0 (3)
20 sec.	1.4 (12)	7.5 (3)	8.4 (4)
30 sec.	1.0 (12)	4.8 (9)	8.0 (6)
1 min.	0.3 (10)	2.2 (11)	3.2 (11)
2 min.	0 (12)	0.5 (11)	1.6 (12)
3 min.	0 (11)	0 (12)	0 (12)
4 min.	0 (12)	0 (12)	0 (11)

ments lasting over 5 minutes, the production of crossvein defects is eliminated and the flies, if they survive, are completely normal. Fig. 5 illustrates the quantitative effects on survival at 42.5°. All treatments at 40.5° discussed here are given 25 hours (at 23°) after puparium formation. Pretreatments are given immediately before. Time lapses of up to 2 hours between pretreatment and treatment are of no great consequence, but after that both the effectiveness and the differential nature of the pretreatment fall off. This is illustrated in Table III.

The relative efficiencies of the various pretreatment temperatures have been compared. Rather surprisingly they are, in general, all quite similar at least with respect to survival; that is to say that a given pretreatment duration at any of the temperatures in the range from 31 to 38° will produce similar changes in resistance to killing at 40.5°. Table IV contains representative data on the resistance conferred by pretreatment of various durations for various

temperatures from 30 to 38°. The very short pretreatments illustrate the differential decrease in crossvein defects and in death. Table V shows the decrease in crossvein response after pretreatment at 36.5° lasting as little as 10 seconds. When pretreatments are as long as 4 minutes, the crossvein response

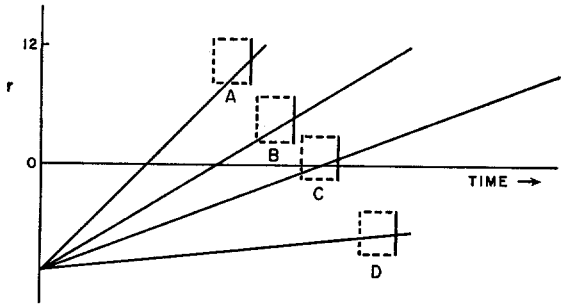

FIGURE 6. Schematic representation of the relationship between resistance to crossvein defect formation and to death conferred by pretreatment at 36.5°. *A*, control. *B*, short pretreatment in which death is deferred by the reduction of the rate of destruction of crossvein-making ability (slope) is such that death precedes maximum formation of defects. *C*, after longer pretreatment death, though deferred even further, still takes place before the production of any but the slightest defects. *D*, death, deferred even further, takes place before any crossvein defects can be observed. Vertical solid line represents the time at which all pupae are killed. The dotted box represents the times at which few to all flies are killed and the degree of crossvein defects produced at these durations.

TABLE VI

COMBINATIONS OF PRETREATMENTS AND MAXIMUM SURVIVED TREATMENTS AT 40.5°

Pretreatment	Duration at 40.5°	Survival
	min.	
1 hr. each at 32°, 35°, 37.5°	135	6/12
10 min. each at 32°, 35°, 37.5°	110	2/12
	100	9/12
	90	9/12
20 min. each at 32.5°, 35°, 37.5°	130	0/24
	120	2/48
	110	9/24
	100	20/72

has been pushed beyond death, as it were. Fig. 6 illustrates this relationship schematically.

It is interesting that these 2 temperature adaptation effects can be combined to produce an even greater resistance to death. Animals may be exposed for several hours or longer to temperatures in the upper physiological range. They

may then receive additional pretreatment in the 31 to 38° range before treatment at 40.5° at the age of 25 hours. Tables VI and VII list the results of various combinations of pretreatments and treatments. Since the combined pretreatments enable the animals to withstand longer exposures to 40.5° than would be possible after any single pretreatment of either type, no matter how great, it must be concluded that the mechanisms of the two kinds of adaptation are different. On the other hand, the fact that they summate enables one to conclude that they are joined in a final common path.

Possible Mechanisms of Temperature Adaptation

It is of interest that the rate of destruction of crossvein-making ability, or the denaturation of the protein at 40.5°, seems to proceed at the same rate in animals raised at 23° and in those raised at 28°. And yet the ones raised at 28° withstand longer exposure. This is illustrated in Fig. 7 which shows the relevant duration-response relationships. According to the reasoning employed

TABLE VII

COMBINED TEMPERATURE ADAPTATION EXPERIMENT

Pretreatment at 28°, then pretreatment at 37.5°, then treatment at 40.5°

Duration at 28°	Duration at 37.5°	Duration at 40.5°	No. treated	No. surviving	Per cent
	min.	*min.*			
18½ hrs. (= 25 hrs. at 23°)	10	80	12	3	25
	10	95	12	0	0
	20	100	36	6	20
	30	100	24	12	50

above, one may extrapolate back to the reserve amount of protein present before treatment. Animals raised at 28° appear to have considerably greater reserves. This is evidence favoring the idea that animals raised at 28° synthesize more of the protein under discussion, and that this is the basis for the increased resistance to the production of crossvein defects at 40.5°. Extensive dosage-response experiments were run on flies aged at 23° and on flies aged at 28°. The slopes of the dosage-response curves were quite similar. On the other hand, the curves for flies aged at 28° extrapolated back to lower intercepts indicating greater reserve crossvein-making ability. These experiments were run twice, a year apart, and each time the results were essentially the same. Table VIII lists the calculated reserve crossvein-making ability for both sexes of flies in both experiments, as well as the slopes of the dosage-response curves. Final applications of this hypothesis and further relevant evidence will be discussed later on.

Because of the relationship between the two forms of temperature adaptation under discussion, one is impelled to look for another mechanism under-

lying the more rapid type. It is conceivable that this mechanism involves the conferring of resistance on the molecular level on the protein already in existence. This contrasts, of course, with the mechanisms proposed immediately above where *more* protein, but with unchanged susceptibility to denaturation, would be produced at higher temperatures in the physiological range. One possible way of conferring resistance to irreversible denaturation upon a pro-

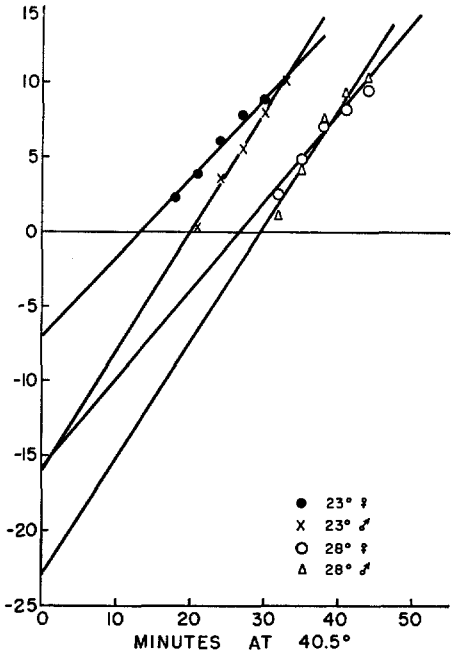

FIGURE 7. Extrapolation of dosage-response curves for flies raised at 23 and 28° Slopes seem similar (for a given sex) but *y*-intercepts are different.

TABLE VIII

DOSAGE-RESPONSE DATA FOR FLIES SPENDING THEIR FIRST PUPAL DAY AT 23° (25 HOURS), AT 25° (23 HOURS), AND AT 28° (18½ HOURS)

Treatment is at 40.5°C

		Experiment I		Experiment II	
Temperature	Sex	Slope	*y*-intercept	Slope	*y*-intercept
°C					
23	Females	0.48	−7	0.52	−7
	Males	0.73	−17	0.79	−16
25	Females	0.50	−11		
	Males	0.75	−18		
28	Females	0.53	−15	0.58	−16
	Males	0.76	−25	0.78	−23

tein is to denature it reversibly first. The effectiveness of temperatures in the 31 to 38° range lends some slight support to the notion that such reversible denaturation is taking place. This is a temperature range at which one would expect sufficient protein denaturation to have an impact on the organism, since it is above the maximum temperature at which the flies can survive. Further support of a stronger variety would be given by the finding of a temperature coefficient consistent with protein denaturation. This matter is under investigation now. Here it is important to distinguish resistance to the production of crossvein defects from resistance to death. The temperature coefficient with respect to conferring resistance to death is near 1. Here, either reversible protein denaturation is not involved, or else a number of different proteins are involved in a complex mechanism. On the other hand, only very short pretreatments, such as have been done at 36.5° (lasting from 10 seconds to 4 minutes), can reveal the relative effectiveness of temperatures in the 32 to 38° range in increasing resistance to the production of crossvein defects.

Summation Experiments

Several kinds of summation experiments can be performed to support the evidence gained through single experiments. Four are relevant here. First, treatments may be split at a given temperature in the 39.5 to 41.5° range. If allowance is made for a 5 minute warm-up period, the split treatments are additive, if the interval between them is not so long as to cause some part of the treatment to be outside of the maximally sensitive period. These experiments support the idea of a constant destruction of crossvein-making ability at these temperatures. The 5 minute warm-up time is also confirmed.

Second, treatments at two temperatures in the 39.5 to 41.5° range can also be summated according to the same rules and using 2.3 as the Q_1. Results of this type of experiment are listed in Table IX.

Pupae were first exposed to the lower temperature and then to the higher of the two that were used. In between, the vials were allowed to return to room temperature. The results may be compared with results expected for a single treatment at an equivalent time at 40.5°. Equivalent times were calculated by subtracting 5 minutes from each of the treatments, converting the remainder to equivalent time at 40.5° (using $Q_1 = 2.3$), and then adding one 5 minute warm-up period to give the equivalent treatment time at 40.5°. Each experiment involved 15 or 30 pupae, and the comparison between the observed and the expected data gives reasonable evidence for the additivity of the treatments.

Third, treatment split between the peak sensitivity period and other times can define the total period of sensitivity pattern more effectively than can single experiments. This is because at times of lower sensitivity the animals

may be killed before crossvein defects are produced; but if a subliminal treatment is given in the peak sensitivity period, the additional exposure at a period of lesser sensitivity may cross the threshold without killing the animal. Fig. 8

TABLE IX

SUMMATION OF TREATMENTS AT TWO TEMPERATURES IN THE 39.5 TO 41.5° RANGE

Treatment 40.0° + duration in min.	Temperatures 41.0°	(Estimated equivalent time at 40.5°)	Average crossvein defect (r)			
			Observed		Expected	
			♂	♀	♂	♀
30	12	(32½)	4.5	5.4	6.0	7.5
30	10	(29½)	3.8	5.5	3.5	6.0
20	12	(25½)	0	2.5	0.5	4.0
20	10	(22½)	0	3.4	0	2.5
39.5° +	*40.5°*					
20	20	(26)	0	0.4	0.5	4.0
40	20	(35)	6.3	7.1	8	9

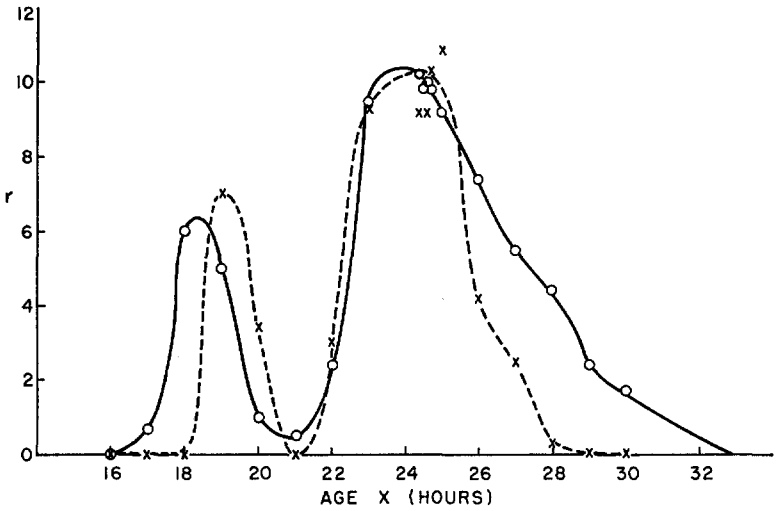

FIGURE 8. Summation experiment. Pupae treated at 40.5° for 20 minutes at 25 hours and 20 minutes at age *X*.

illustrates the use of such experiments. As can be seen by comparison with Fig. 1 *a*, this method is more sensitive.

Fourth, treatments in the 37.5° range do not summate with treatments in the 39.5 to 40.5° range, thus supporting the idea that there is no final common path to these mechanisms. Indeed prior exposure to 37.5° antagonizes treatments at higher temperatures. It was through these experiments that the rapid temperature adaptation in the range eventually extended to include tempera-

tures from 31 to 38° was first found. Posttreatment at 37.5° does not nullify the response, however. On the contrary when one treats in the 39.5 to 41.5° range first, and then adds a treatment at a lower temperature, the treatment at the lower temperature adds to the results. It has been seen repeatedly that prior treatment at 39.5 to 41.5° makes subsequent treatment at lower temperatures, at least down to 37.5°, behave as do temperatures in the 39.5 to 41.5° range. Although this "sensitization" of the pupae to lower temperatures has been demonstrated in numerous cases and without relevant exception, no systematic investigation of the kinetics has yet been made.

Modification of Association between Qualitative Effects and Given Temperatures

As has been stated above, the association between the qualitative effect and a given temperature range holds only for treated animals who have spent their early pupal life at 23°. For example, holes in the wings are produced by temperatures in the range from 37.3 to 37.8°. The fact that it is impossible to produce holes in the wings of such animals at temperatures either above or below this range gives a qualitative overtone to this range. It is, therefore, most interesting that animals raised at 28° respond in this manner to a different temperature range, that from 38.0 to 38.2°. In short, it would appear that these effects of temperature are more than effects of degree, and that in a given context each temperature, above the physiological range at least, is associated with the organism in a qualitative manner. Other effects of temperatures in this similar range are also seen to depend upon the temperature at which the flies spent their early pupal life. Fig. 9 illustrates the relationship of crossvein defect production to temperature in the 36 to 38° range for flies raised at 28°. The displacement of the peak responses with respect to temperature is clear, though not as striking as the case of the holes in the wings.

In the range from 39.5 to 41.5° it is impossible to distinguish a qualitative relationship from a merely quantitative one. Animals raised at 28° require longer exposure to a given temperature to produce crossvein defect. By itself this information might be interpreted as evidence for increased general resistance to thermal effects brought on by temperatures in the upper physiological range. On the other hand, one might consider that animals raised at 28° "interpret" temperatures as being several tenths of a degree lower than they would have if they had been raised at 23°. Although this idea ordinarily would not come to mind, in the light of the previous qualitative examples it is worth considering.

Generality of the Temperature Effects

At the time of pupation the animals are more sensitive to heat shock than they will be until the period just before adult emergence. For the intervening time a fairly uniform sensitivity is observed. Forty-five minutes at 40.5° will kill

almost all the animals at any age during this period. Similarly, response to various exposures to various other temperatures is fairly uniform all during this period to this extent: the amount of exposure needed to kill remains about the same, and that needed to produce one phenocopy or another also remains the same.

In addition pretreatments at temperatures in the 31 to 38° range bring about increased resistance at any time during this period as they do at the age of 25 hours. Finally, the effects of temperatures in the higher physiological range are seen at all times during this period of pupal development. This implies the existence of two general mechanisms of temperature adaptation.

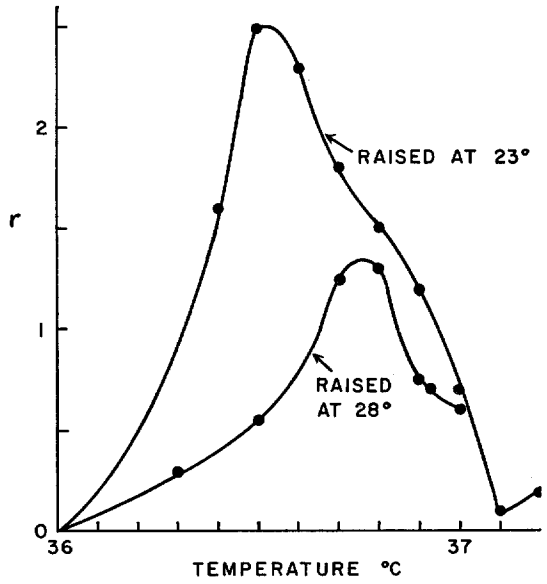

FIGURE 9. Displacement of temperature-response curve for production of posterior crossvein defects in 36 to 37° range when pupae are raised at 28°.

Genetic Variation

It is known from the work of Waddington (13, 14), Milkman (6–8, 10), Bateman (2), and others that the development of posterior crossvein is affected by numerous genes. In addition to the single genes which, as dominants or recessives, cause defects in the posterior crossvein, there are a number of extremely common genes with individually minor effects on the development of this vein. Flies carrying a few of these genes may be more susceptible than usual to the production of crossvein defects by heat shock. In other words, small numbers of these genes make a visible difference only under certain environmental conditions. When larger numbers of these genes are brought together in a single fly, no heat shock is necessary to produce the crossvein

defects. It might be expected, therefore, that a good deal of natural variation in susceptibility to the production of the various defects discussed above would be observed. In fact this is not the case. Of twenty-one different strains of flies, each derived from a single wild inseminated female, all showed comparable responses to various temperatures in the ranges described above (10). Indeed various flies carrying mutant genes that made them extremely abnormal morphologically (genetic marker strains) also responded in much the same way. This would imply that the combinations of genes generally found in flies are equivalent in their effects on response to the various heat shocks, although

TABLE X
EFFECTIVE TEMPERATURES OF SOME *DROSOPHILA* SPECIES

Species	Killing at 35 min.	Posterior crossvein defects	Anterior crossvein defects	Q_1 of killing in upper range
D. busckii	39°	34.5 to 35.5°	34.5°, 35.5°	Around 2
*D. funebris**	37.5°	36–37.5°	37.5°, 38.0°	Around 2
D. hydei	40°	None	None	Around 2
D. melanogaster	41°	36–37°, 37.3–37.8°, 39–42°	38°	2.3
D. pseudoobscura	39°	35.5°	35.5°	Around 2
D. virilis‡ (raised at 28°)	42.5°	39.0–42.5°	40°, 40.5°	2.3
D. willistoni	39°	35°, 38°, 29°	35.5°, 36.5°, 38°, 39°	Around 2

* Data of Kathleen Peterson.
‡ Data of Charles Martin, Margaret Castle, and Dr. A. T. Collette.

genetic combinations may be synthesized by selection which alters these responses. This difference is analogous to the difference between a gin rummy hand as dealt and after a few cards have been played.

Species Variation

The development of posterior crossveins seems to be a relatively sensitive process in a number of species of *Drosophila.* In wild individuals the posterior crossvein is one of the more variable structures, and defects are observed rarely, but repeatedly, in various species. Moreover, several species have been shown to respond to heat shock in much the same way as *D. melanogaster* pupae do. The species tested so far are *D. busckii, D. funebris, D. pseudoobscura, D. virilis,* and *D. willistoni.* Attempts to produce crossvein defects by heat shock were successful in each of these species and the details are given in Table X. Extensive attempts to produce crossvein defects in *D. hydei* have so far failed completely. In addition to crossvein defects, other defects can be produced in various species, some of which are not general to the genus. For example,

although it has been impossible frequently to produce longitudinal vein defects by heat shock in *D. melanogaster* (but *cf.* reference 5), these are easily produced in *D. virilis.*

DISCUSSION

It has long been tempting to consider protein denaturation as a likely intermediary in the morphological effects of high temperatures on organisms, particularly developing organisms. Two kinds of circumstantial evidence support this idea. One is simply the importance of proteins, particularly enzymes, in development. The other is the general association of lethal temperature ranges with the ranges in which protein denaturation becomes considerable. Now, the production of posterior crossvein defects in the range from 39.5 to 41.5° at 25 hours after puparium formation seems in its kinetics to support the idea of the role of protein denaturation. The aspect of posterior crossvein formation here affected is unusual in its degree of differential sensitivity, for it has been possible to analyze the disturbance of this process to an unusual degree. This was possible because the treatments over a wide range did not visibly disturb any other relevant processes. The consistency of the temperature coefficient measurements, of the dosage-response relationships, and of the summation experiments, internally and with one another, gives one confidence in the validity of the analysis.

None of the other temperature effects (*e.g.* production of crossvein defects at 36.5 and 37.5°) can be analyzed as easily in comparable detail. Nevertheless, I propose to consider the various other temperature effects as being mediated by protein denaturation also, with the purpose of seeing what testable conjectures about biological organization can be made.

One is struck by the fact that various responses do not necessarily increase with temperature. But there are optima of derangement, as it were, which are islands of abnormality in an otherwise normal range. It may be added that even death itself can appear on such an island, for treatments for 4½ hours at 37.5° kill essentially all the males, whereas treatments of similar duration at 38.0° do not. One is forced, therefore, to consider an individual sensitive process not as a labile molecule, but rather as a system whose balance must not be disturbed.

Life at varying temperatures is made possible by the fact that the living processes manage to keep in step with one another. The temperature coefficients of various enzymes in an organism are similar, and it is a general rule that many of the various enzymes of a given organism are roughly comparable in their resistance to thermal denaturation. One might visualize the temperature-rate relationships as a series of parallel strands of rope. But more than this holds the rope together. Undoubtedly feedback and equilibrium mechanisms, including such things as product and substrate concentrations, keep

the strands braided. At a lethal temperature, the strands fly apart. And yet we have seen that the various effects are not restricted to the very end of the rope. There are, to carry the image a little further, several places near the end where the strands begin to come apart, only to come together again. These are the places where individual processes are particularly sensitive. At these temperatures one may find specific morphological effects intermediate between normal development and death. One lead that might be of considerable general importance is provided by the finding that animals raised at 28° are more resistant to exposure to 40.5° than animals raised at 23°. This increased resistance is associated with an apparent higher concentration of the protein whose denaturation at 40.5° leads to crossvein defects. Two facets of this finding are of particular interest. One is that the resistance conferred by raising at 28° has to do both with crossvein defects and with death; the other is that resistance to death is higher throughout the whole pupal life. That this increased resistance should be associated with increased amounts of protein concerned with crossvein formation on one hand, and that similar resistance should be encountered throughout pupal life lead one to consider the possibility that one general mechanism of temperature adaptation is a general increase in protein synthesis and, therefore, in the amounts of protein present. This might be merely a relative increase in protein concentration (animals raised at higher temperatures are often smaller) or it might be an absolute increase. At any rate this possibility is simply tested. The comparative analysis of the total protein in various organisms at various ages raised at temperatures high and low in their physiological range is now under way. Results so far imply that there is no difference in total protein or in total soluble protein, between samples of pupae raised at 23 and at 28° for their first biological day of pupal life. The idea of a *general* effect on protein synthesis is therefore not supported.

The rapid temperature adaptation consequent upon treatment in the 31 to 38° range is more to be marveled at than discussed. But if indeed reversible protein denaturation is protecting against irreversible protein denaturation, this mechanism might conceivably be brought to light by histochemical experiments, as well as by the temperature coefficient experiments now under way. The effects of modifying secondary and tertiary structure, at various pH's and ionic strengths, on sensitivity to thermal denaturation are well known. More information on this subject should find diverse uses (1).

The technical assistance of Mrs. Knut Samuelson and Mr. Donald Phillips, and the clerical assistance of Mrs. William Heyl are gratefully acknowledged.

This work was supported by Grant G-14722 from the National Science Foundation, with these exceptions: the work on species other than *D. melanogaster* and *D. virilis* and the publication costs were supported by National Institutes of Health Grant RG-7810 (C_1).

Received for publication, November 9, 1961.

REFERENCES

1. ABELSON, P. L., Extra-terrestrial life, *Proc. Nat. Acad. Sc.*, 1961, **47**, 575.
2. BATEMAN, K. G., The genetic assimilation of four venation phenocopies, *J. Genetics*, 1959, **56**, 443.
3. BULLOCK, T. H., Compensation for temperature in the metabolism and activity of poikilotherms, *Biol. Rev.*, 1955, **30**, 311.
4. DAVSON, H., A Textbook of General Physiology, Boston, Little, Brown and Company, 1959.
5. MA, SUNG-YÜN, Experimentelle Untersuchungen über Hitzemodifikationen des Flügels von *Drosophila melanogaster*, *Arch. Entwcklngsmechn. Organ.*, 1943, **142**, 508.
6. MILKMAN, R. D., The *crossveinless* complex, a genetic system in natural populations of *Drosophila melanogaster*, Ph. D. Thesis, Harvard University, 1956.
7. MILKMAN, R. D., The genetic basis of natural variation. I. Crossveins in *D. melanogaster*, *Genetics*, 1960 *a*, **45**, 35.
8. MILKMAN, R. D., The genetic basis of natural variation. II. Analysis of a polygenic system in *Drosophila melanogaster*, *Genetics*, 1960 *b*, **45**, 377.
9. MILKMAN, R. D., The genetic basis of natural variation. III. Developmental lability and evolutionary potential, *Genetics*, 1961, **46**, 25.
10. MILKMAN, R. D., The genetic basis of natural variation. IV. Natural distribution of the *crossveinless* polygenes of *Drosophila melanogaster*, *Genetics*, 1962, in press.
11. PROSSER, C. L., Physiological variation in animals, *Biol. Rev.*, 1955, **30**, 229.
12. PROSSER, C. L., Comparative Animal Physiology, Philadelphia, W. B. Saunders Company, 2nd edition, 1961.
13. WADDINGTON, C. H., Genetic assimilation of an acquired character, *Evolution*, 1953, **7**, 118.
14. WADDINGTON, C. H., The Strategy of the Genes, 1957, New York, Macmillan Company, 1957.