On the Mechanism of Some Temperature Effects on *Drosophila*

ROGER MILKMAN

From the Department of Zoology, Syracuse University, Syracuse

ABSTRACT The results of detailed kinetic studies on temperature adaptation and on crossvein deformation in *Drosophila melanogaster* pupae are presented. A scheme which unifies most of these effects is offered, suggesting that the basic events are a series of changes in the tertiary structure of a protein. Implications of the findings and the scheme are discussed with particular respect to temperature adaptation and to development.

INTRODUCTION

Formation of the posterior crossvein in the wing is a sensitive developmental process in a number of *Drosophila* species. It may be disturbed by the presence of certain alleles at a large number of genetic loci (2, 4, 8, 10-12) and by exposure to various high temperatures (5). Disturbance of posterior crossvein formation can be measured quantitatively in terms of the fraction missing. Since the alleles which influence it are so numerous, this characteristic has been valuable in the determination of natural genetic variation.

Exposures, prior to treatment, to temperatures in and above the physiological range also influence the response, so that it is a good end point for temperature adaptation studies. Previous investigations of the effects of high temperatures on organisms (3, 5, 7, 9) have indicated that the thermal history of an organism is generally highly significant in determining its resistance to such effects and that the alteration of protein structure may be of widespread importance in mediating these effects. In this paper an attempt will be made to show how various temperature effects on the formation of the posterior crossveins can be related to one another and possibly to the alteration of tertiary structure in proteins.

METHODS

The methods to be described work for a variety of *Drosophila* species. In order to be specific about details, only *Drosophila melanogaster* will be discussed.

Animals are collected at the time of pupation, a stage which lasts for about an hour, and which therefore provides an accurate point in time from which the age

of animals can be measured. Twenty-five hours after puparium formation at 23°, or at a corresponding biological age at other temperatures, the pupae respond to exposure to certain high temperatures by being unable to form their posterior crossveins normally. The posterior crossveins are linear structures, and defects can be measured quantitatively by dividing each crossvein into imaginary sixths. Since there are two posterior crossveins, one on each wing, the scale of crossvein defects is in twelfths. A class of flies can be characterized by the average response, from "0" (normal) to "12" (crossveins completely absent).

Treatments are made in Precision water baths controlled to within 0.1°C or less. The pupae are either in shell vials (in which case the warmup time is 2 to 5 minutes depending on type and thickness) or in teabags (warmup is essentially instantaneous, less than 2 seconds). The pupae will stand immersion in water for about 40 minutes with no major relevant side effects. Treatments are given either singly or in various combinations. We shall speak of *pretreatments*, which may modify subsequent treatments. The *interval* between pretreatment and treatment may be spent at a temperature in the physiological range or at a high temperature. The *treatment* is the final exposure to high temperature and starts 25 hours after puparium formation in most experiments.

Basic information, such as response *vs.* age, response *vs.* duration, temperature coefficient of the over-all response, effective temperature range, and effects of temperature at which the animals spend their 1st day of pupal life, has been published previously (5).

To summarize, 25 hours after puparium formation (23°) posterior crossvein defects may be produced in the temperature range from 39.5 to 41.5° according to the following dosage-response relationship:

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

where r = the average crossvein defect rating. k is a constant dependent on sex. For males $k \cong 0.75$, for females $k \cong 0.5$. t = dosage in minutes, T_t = treatment temperature (°C), and f is a function, different for each sex and as yet otherwise undescribed. T_a is the temperature (°C) at which the animals spend their 1st pupal day. The observable duration-response curve is, therefore, a linear function with a Q_1 of 2.3; and $f(T_a)$, the y-intercept, defines the amount of crossvein-making ability in excess of normal need. This empirical relationship will be modified somewhat in the light of experiments to be described.

Highly inbred flies of the Oregon R strain were used exclusively. Treatment durations are stated after subtracting warmup time.

OUTLINE OF PHENOMENA TO BE DISCUSSED

In addition to the dependence of response upon the temperature in the physiological range at which the animals spend their 1st day of pupal life, short pretreatments at high temperatures strikingly antagonize the production of crossvein defects by subsequent exposures to high temperatures. Analysis of this latter form of rapid temperature adaptation has led to the resolution of

three distinct processes, all of which will be described. These processes seem to be related sequentially to one another and to the process which ultimately results in the posterior crossvein defects. The scheme resulting from this analysis ties together a number of different high temperature effects on the pupae.

Two other characteristics of the production of posterior crossvein defects at high temperatures are also relevant. The first is that it is essentially impossible to produce posterior crossvein defects with a single treatment at 38.5°, although in view of the length of the sensitive period and the temperature coefficient obtained between 39.5 and 41.5°C, it should easily be possible to do so. Second, if part of the treatment is given in the range from 39.5 to 41.5°,

TABLE I

RESULTS OF SPLIT TREATMENTS AT 40.5° WITH VARIOUS
INTERVALS AT ROOM TEMPERATURE

Duration of first treatment	Interval	Interaction of first treatment with second
18 min. or more	0–6 hrs.	Additive
2–12 min.	5 min.	Additive
	2 hrs.	Antagonistic
30 sec.–1 min.	5 min.	Antagonistic
	2 hrs.	Antagonistic
10 sec.	5 min.	Antagonistic
	2 hrs.	Not antagonistic

subsequent treatments at temperatures down, at least, to 32.5° act additively and produce crossvein defects. By themselves, of course, treatments at these lower temperatures would not be effective (two exceptions are considered in the Discussion). These observations can also be related to the common scheme.

RESULTS

Split Treatments

A general idea of the sequence of temperature effects may be gained from Table I. A single 35 minute treatment at 40.5° produces extreme crossvein defects (average rating, 9) in both sexes. If this effect were due to a single process, one would expect split treatments to be additive whether they were split equally or unequally. As has been reported previously (5), split treatments do act additively when the first part of the treatment at 40.5° is 20 minutes or longer. On the other hand, when the first part is shorter, different results are observed. These are summarized in Table I.

It will be seen in this table that after 18 minutes at 40.5° the pupae have been affected unalterably. No matter what the interval between the split parts of the treatments, the effects of the first 20 minutes cannot be undone. (Of course, these treatments must be made in the sensitive period, and the quantitative changes in sensitivity throughout this period must be taken into account.)

Splitting the treatment unequally, with the first part lasting 12 minutes or less, produces an array of strikingly different results. The dependence of these results on the interval between the two treatments can also be seen. One must conclude that several events are taking place over the course of the total 35 minutes at 40.5°, and that the course of changes can be diverted by lowering the temperature for certain durations at certain times. The analysis of these changes has been effected through a series of experiments which will now be described.

Pretreatments of the First Type

Very short pretreatments at 40.5°, separated from the treatment by a short interval, result in the reduction or prevention of the appearance of posterior crossvein defects. Pretreatments as short as 10 seconds with an interval at room temperature of 10 minutes render a subsequent treatment lasting 35 minutes or 43 minutes at 40.5° completely ineffective, although 35 minutes alone would ordinarily produce extreme crossvein defects, and 43 minutes would kill all the pupae before adult emergence. Shorter intervals reduce this antagonistic effect of the pretreatment. It is therefore concluded that two changes are taking place. We may speak of changes in the tertiary structure of a single protein (evidence will be adduced for this) or, more conservatively, merely of changes in state of the pupa. In any case, starting with structure or state "A," the short exposure to 40.5° induces a change to "B." A subsequent interval at room temperature converts B to "C," which is resistant to the effects of subsequent treatment at 40.5°. It is necessary to think of two changes, since two separate temperature effects are involved. The A to B conversion has been demonstrated at a number of temperatures. Table II shows the durations of some of these temperatures required to produce a standard degree of protection with a 5 minute interval between pretreatment and treatment. The temperature coefficient calculated from these data is 1.4 for a degree ($Q_1 = 1.4$), corresponding to an activation energy of about 70,000 cal/mol. This is in the range required for a change in the tertiary structure of a protein, and no other known relevant chemical process with such a high activation energy occurs in *Drosophila* in this temperature range. This is the sole evidence that the change is really one in protein structure, and, of course, it does not constitute proof. The temperature coefficient of the B to C conversion seems to be about one in the range from 18 to 28°.

With intervals longer than 30 minutes, the protective effect gradually wears off, indicating the conversion of C back to a susceptible state. Fig. 1 shows the response to 35 minutes at 40.5°, given at 25 hours, after various pretreatments and intervals. It will be noted that protection declines (re-

TABLE II

TRANSIENT RAPID TEMPERATURE ADAPTATION
AT VARIOUS TEMPERATURES

Pretreatment			Response	
Temperature		Duration	Male	Female
°C				
28.0		5 min.	8.7	7.8
	→	10	1.3	3.9
		15	0.4	1.6
		20	0.8	0.8
32.0		1½	3.1	6.5
	→	2	0.8	4.0
		3	0.0	1.4
		6	0.0	0.0
34.5		10 sec.	5.0	7.9
		20	5.0	8.0
		30	4.1	8.0
	→	1 min.	0.7	4.9
		1½	0.0	0.8
		2	0.0	0.5
36.5		5 sec.	3.5	7.3
		10	5.3	7.6
		20	1.9	5.4
	→	30	1.8	3.3
		1 min.	0.5	2.7
		1½	0.1	0.6
		3	0.0	0.0

Response measured in average posterior crossvein defect (range 0 to 12). Interval at room temperature, 5 minutes. Treatment, 35 minutes at 40.5°. Sample per treatment, 20 to 100 pupae. Arrows indicate pretreatments giving comparable protection. The time at these temperatures ostensibly permits partial conversion of A to B.

sponse rises) with increasing intervals after the very short pretreatments. Table III shows that this decline in protection is due to the length of the interval, not to the pretreatment age. It should be noted that some reduction in response must be expected when the treatment comes at 26 or 26½ hours. In spite of this, it is seen that the response to 35 minutes at 40.5° is greater 60 and 90 minutes after pretreatment at 25 hours than it is 30 minutes after

the same pretreatment. Similarly, with pretreatment at 23 or 24 hours, protection is complete after 30 minutes but incomplete after 60 and 90.

Pretreatments of the Second Type

If the pupae are pretreated for longer times (about 30 seconds) at 40.5°, protection is also conferred against subsequent treatments at 40.5°. The interval required between pretreatment and treatment is approximately the same as that described in the previous case, but this protection in contrast
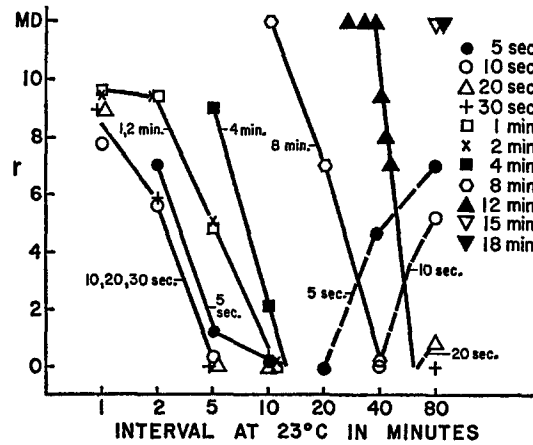

FIGURE 1. Response to 35 minutes at 40.5° (age, 25 hours) after various pretreatments and intervals. Each curve represents a pretreatment duration at 40.5°, or a group of such durations. Note the rising dotted lines, which show how the protection conferred by the shortest treatments wears off. MD = mostly dead. Data are for females only.

TABLE III

PRETREATMENT AGE, INTERVAL LENGTH,
TREATMENT AGE, AND RESPONSE

Interval length		Treatment age, hrs.						
		23½	24	24½	25	25½	26	26½
min.								
30	Males	0.1		0.0		0.0		
	Females	0.0		0.1		0.0		
60	Males		0.9		2.0		1.0	
	Females		2.8		3.4		2.0	
90	Males			0.9		1.8		0.9
	Females			3.2		2.7		0.9

Pretreatment, 10 seconds at 40.5°C. To find pretreatment age, subtract interval length from treatment age. Treatment duration, 35 minutes.

to the form described above does not wear off. Also the temperature range in which the interval may be spent is greater, extending from 18° (and perhaps lower) to 38.5°. It is therefore concluded that this effect results from the conversion at 40.5°C of B to "D," which in turn is converted to "C'" in the interval between pretreatment and treatment. In order to distinguish protection of the first type from protection of the second type, the pupae may be pretreated 21 hours after puparium formation. Since C returns to a suscepti-

TABLE IV

LASTING RAPID TEMPERATURE ADAPTATION
AT VARIOUS TEMPERATURES

Pretreatment at 21 hrs.		Response to 35 min. at 40.5° at 25 hrs.	
Temperature	Duration	Males	Females
°C			
—	0	10.6	9.6
32.5	20 sec.	8.6	9.8
	30	8.4	9.3
	40	9.8	10.0
	1 min.	8.7	9.5
	2	8.0	8.5
	5	8.2	8.8
	10	4.5	9.7
	15	1.1	4.3
→	20	0.7	3.6
	25	0.0	0.7
	30	0.0	1.1
36.5	5 sec.	9.2	9.7
	10	(6.5)	10.0
	20	(5.0)	9.4
	30	8.3	9.1
	40	4.5	8.8
	1 min.	2.8	6.8
→	2	0.4	4.7
	3	0.0	1.1
	4	0.0	0.0
	5	0.0	0.9
	10	0.0	1.2
	20	0.0	0.0
40.5 →	10 sec.	0.3	3.5
	20	0.0	0.0
	30	0.0	0.0
	1 min	0.0	0.0

See Table II for explanation. Durations at these temperatures ostensibly permit partial conversion of B to D. Interval 3½ to 4 hours, depending on duration of pretreatment. This permits conversion of D to C'.

ble state long before 3 hours have elapsed, any protection against treatments at 25 hours must result from the formation of C'. Protection of this type may again be produced at various temperatures. Table IV illustrates the durations at various temperatures necessary to produce comparable protection. From these data the temperature coefficient of 1.8 for a degree ($Q_1 = 1.8$) emerges. This corresponds to about 110,000 cal/mol, which is also in the range commonly seen for change in the tertiary structure of a protein. Two changes are once more required to explain the two additional temperature effects. Moreover, C' must be different from C because it does not return to a susceptible state, and, therefore, D must be different from B. The Q_1 of 1.8 refers to the two-step process A→B→D, but it is probably a fairly good approximation of the Q_1 for B→D, since this is the slower step.

It is noteworthy that protection of this form may be conferred as early as the time of puparium formation. In fact, a 2 minute pretreatment at this time will protect strikingly against the production of crossvein defects by exposure to 40.5° at the peak sensitivity period 25 hours later. This indicates that the protein involved (if that is what it is) is present in good quantity at a time when the developing wing is little more than a very large imaginal disc. Shorter treatments than 2 minutes at this time give less protection. Perhaps the longer duration required at this early stage is a reflection of a lesser amount of protein being present than is formed later; alternatively it may reflect differences in the protein's environment. At all times intermediate between puparium formation and treatment time, protection may be conferred in this way. Preliminary experiments indicate that late third instar larvae can be protected to a lesser extent.

Pretreatments lasting 1, 2, and 4 minutes provide less protection than 30-second pretreatments when the interval is up to 10 minutes long (Fig. 1). This is particularly clear in the upper range of interval temperatures (see also Table VIII). This implies that the amount of D is decreasing, and so the amount of C', the protected state, that can be formed during the interval is progressively reduced. The reduction in the amount of C' formed is thus related to the lowered protection. The reduction in quantity of D must be coincident with the formation of the next state in the series, which we shall call "E" and describe below.

It should be pointed out now that the events described as a series do overlap to a considerable extent. Thus the peak amount of a particular state is not found just as the last bit is being formed. It appears that the peak amount of B occurs at about 7 seconds (40.5°). At 10 seconds, some D is clearly present (Table IV). The D peak is likely to be around 20 seconds.

Pretreatments of the Third Type

Pretreatments at 40.5° lasting 2 to 12 minutes impose two important changes from the A state. One is the now familiar protection; the kinetics are different,

but a sufficient interval at room temperature will prevent or reduce the inter-
ference with crossvein formation by a subsequent treatment at 40.5°. It will
be shown that this protection cannot be attributed simply to the conversion
of D to C'.

The second change is that crossvein defects may now be produced by sub-
sequent exposure (with little or no interval) to temperatures ranging down to
32.5° and perhaps below. The required duration of such a further treatment
depends upon the temperature: the Q_1 is about 1.5. Table V shows the sum-
mation between treatments at 40.5° and subsequent treatments at lower
temperatures. This new state is called E, and it is converted to C″ at room
temperature.

Fig. 2 shows the response to 90 minutes at 38.0° after various durations at
40.5°. The sharp early rise in the curve is thought to reflect the production of
E from D. Only E will go on to the non-functional state at this temperature.
Subsequently, the curve rises more slowly: this is simply the dosage-response
relationship pertinent to 40.5° and reflects merely the greater over-all dura-
tion. It is interesting to see that the inflection point comes at about 5 minutes.
This is when all the D has been converted, ostensibly. But the pupae can still
be protected to a measurable extent after up to 12 minutes, so that E, too,
must be convertible to a more resistant state.

Longer Pretreatments

Pretreatments at 40.5° lasting longer than 12 minutes act additively with
subsequent treatments, whether or not an interval at 23° is inserted. This
means that E is essentially absent after 12 minutes. It has been converted to
"F," which cannot be converted to a protected state. F, however, is not the
final, non-functional state, because crossvein defects do not appear at this
time. We can identify the onset of crossvein defects with the conversion of F
to "G," the final, non-functional state. Of course, it is possible that the true
state of affairs is even more complicated, but this scheme accounts for all of
the present observations. It is illustrated in Fig. 3 and Table VI.

Over-All Temperature Coefficient for the Production of Crossvein Defects

Since the Q_1 of the over-all reaction which produces posterior crossvein de-
fects is 2.3, one must look for one of two things. A *limiting reaction with a tem-
perature coefficient of 2.3* would impose a similar temperature coefficient on the
over-all scheme. Clearly, such a reaction would have to be the slowest one.
The E to F and F to G conversions, which together take almost 90 per cent
of the total time, are clearly the slowest reactions, and the Q_1 of each is only
about 1.5. None of the other temperature coefficients is as high as 2.3 either,
but even if some were, they could not play a significant role in the determina-
tion of the over-all temperature coefficient.

TABLE V

SUMMATION OF TREATMENTS AT 40.5°
AND LOWER TEMPERATURES

First treatment		Second treatment		
			Response	
Duration at 40.5°	Lower temperature	Duration	Males	Females
min.	*°C*	*min.*		
8	38.5	40	0.8	5.3
		50	2.4	7.0
		60	7.5	9.3
		70	10.3	10.0
13		0	0.0	0.3
		30	0.3	5.2
		35	2.1	7.9
		40	4.4	8.5
		45	7.0	8.7
		50	7.0	9.6
		55	9.0	10.2
		60	10.5	10.5
8	36.5	50	0.0	0.2
		70	0.2	3.9
		90	0.8	3.6
		110	3.1	8.2
		120	7.3	8.4
		130	6.1	8.5
13		30	0.0	0.3
		50	0.4	4.2
		60	1.2	6.1
		70	1.2	7.4
		80	1.9	6.8
		90	6.2	9.1
		100	9.4	10.0
18		10	0.0	2.8
		20	0.1	3.0
		30	0.8	4.0
		40	0.5	6.0
		50	3.0	7.0
		60	5.4	8.6
		70	6.2	8.2
		80	8.6	9.8
		90	9.2	9.7
		120	10.0	10.3
23	38.5	5	2.2	4.6
		10	3.2	4.9
		15	5.5	5.1
		20	8.0	8.6
		25	9.1	8.9

TABLE V—*concluded*

First treatment		Second treatment		
			Response	
Duration at 40.5°	Lower temperature	Duration	Males	Females
min.	*°C*	*min.*		
23	37.5	8	1.8	4.6
		15	3.8	5.4
		22	5.5	6.0
		30	6.9	7.8
		38	8.6	8.8
23	36.5	10	1.6	3.9
		20	2.3	4.5
		30	3.6	4.5
		40	5.8	6.5
		50	7.4	7.4
		60	8.0	8.3
23	35.5	15	2.2	4.7
		30	3.4	5.2
		45	5.6	6.2
		60	7.1	7.6
		75	7.5	8.1
23	34.5	20	1.7	4.0
		50	4.2	6.3
		80	6.1	7.1
		110	8.7	8.5
23	33.5	30	1.8	4.6
		75	3.8	5.3
		120	6.2	6.9
		160	7.9	7.6

The over-all temperature coefficient cannot be explained on the basis of the individual temperature coefficients alone. Failing this, it is necessary to look for some *multiplicative relationship*. Such relationships are not numerous, but a very simple one is the following:

$$\frac{dF}{dt} = k(E), \quad \text{or, since E→F is not limiting,} \quad \frac{dG}{dt} = k'\ (E)$$

This relationship would determine the amount of G present after treatment for a certain duration. Since the production of F depends not only on the absolute rate of conversion from E to F, but also on the concentration of E, one might look for temperature dependence in concentration of E. In the scheme described, this would be based on the relative use of the two pathways at the

D branch point, that is, on the relative amounts of conversion from D to C' and from D to E. After 5 minutes at 40.5°, E has reached its maximal concentration. This does not mean that all the D has converted to E. Some has gone to C'. But the D is exhausted, and so no more E will be made. Perhaps at
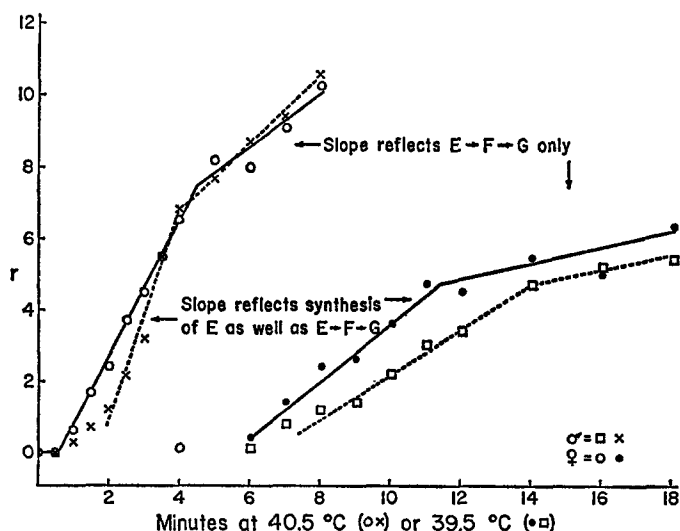

FIGURE 2. Contribution of short early treatment at 40.5° or at 39.5° to response when followed by a fixed subsequent treatment (90 min.), at 38.0°. This graph illustrates the formation of E, the reaction which imposes the temperature threshhold on the production of posterior crossvein defects by the mechanism under discussion.

$$A \rightarrow B \rightarrow D \rightarrow E \rightarrow F \rightarrow G$$
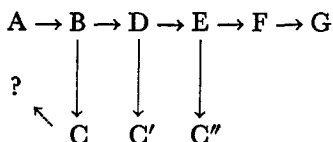

FIGURE 3. Summary of the described changes in tertiary structure of a protein (or in some undefined state of the pupa).

39.5° the amount of E formed is lower and at 41.5°, higher. This possibility has been tested and supported by the following experiment: Some pupae were treated first for 15 minutes at 40.5° and then for 30 minutes at 39.5°. Others were treated first for 30 minutes at 39.5° and then for 15 minutes at 40.5°. This simple pair of reciprocal procedures is lodged in a context that must be reviewed.

Since the total treatment, given without interval, is the same in each case, one might expect identical results. Or, since warmup time must be subtracted from a 40.5° treatment on one hand and from a 39.5° treatment on the other, the latter sequence might produce slightly greater crossvein defects. But if the

total amount of E formed is temperature-dependent, then a striking difference is to be expected; for the first treatment is in each case more than long enough to see all the D converted to its two products, C′ and E. The reciprocal procedures will lead to different responses only if the amounts of E produced are different. The results are seen in Table VII. These data support the previously calculated value for the Q_1 of E→F→G, 1.5. Thus, if the over-all temperature coefficient is 2.3, then $2.3/1.5 = 1.5$ times as much E must be produced at 40.5°C as at 39.5°. These findings also support the idea that the branch point at D is a critical one in determining the concentration of E available for the final conversion to G and concomitant loss of crossvein-making ability.

TABLE VI

A. Characteristics of postulated states

A	Functional	Not protected	Lasting
B	Functional (?)	Not protected	Transient
C	Functional	Protected	Transient
D	Functional (?)	Not protected	Transient
C′	Functional	Protected	Lasting
E	Functional (?)	Not protected	Transient
C″	Functional	Protected	Lasting
F	Functional	Not protected	(Lasting at room temperature)
G	Non-functional	—	Lasting

B. Characteristics of postulated conversions

Conversion	Cumulative time at 40.5°	Known temperature range	Q_1
A–B	10 sec.	28–41.5°	1.4
B–D	30 sec.	28–41.5°	1.8
D–E	5 min.	39–42.5°	?
E–F	12 min.	34.5–42.5°	1.0–1.5
F–G	35 min.*	32.0–42.5°	1.5

Conversion	Duration at 23°	Known effective temperature range	Q_1
B–C	5–10 min.	18–28°	About 1
D–C′	5–10 min.	18–38.5°	Near 1
E–C″	10–20 min.	18–35.5°	—

C. Pathways at constant temperatures

Temperature	Pathway
39.5° and up	A B D E F G
30–38.5°	A B D C′ and A B C (-A?)

* Sufficient conversion to produce extreme defects. Proportion of G formed is unknown.

Although the over-all Q_1 is 2.3, it can be seen that this temperature coefficient is a compound one deriving from the multiplicative relationship of two temperature-dependent factors: the initial concentration of E and the rate at which E is converted to G. Nonetheless, the individual temperature coefficients thus far measured on the main line of the scheme all indicate changes in the tertiary structure of protein.

TABLE VII

A. Results of reciprocal treatments

First treatment*	Second treatment	Response (r)	
		Males	Females
15 min. at 40.5°	30 min. at 39.5°	6.0	6.2
30 min. at 39.5°	15 min. at 40.5°	1.3	2.6

B. Equivalent durations and expected results according to various hypothetical Q_1's for E-F-G

Q_1	First treatment	Equivalent time*	Expected response‡	
			Males	Females
1.5	40.5°	15+20=35	4.9	7.4
2.0	40.5°	15+15=30	1.3	5.0
2.3	40.5°	15+13=28	0.0	4.0
1.5	39.5°	30+22½=52½	(−2)	3.0
2.0	39.5°	30+30=60	0.5	4.5
2.3	39.5°	30+35=65	2.0	5.5

* Including warmup. Subtraction of warmup time leads to moderate differences in expected response to the reciprocal procedures even when the concentration of E is assumed to be temperature-independent, namely when the Q_1 is assumed to be 2.3. Note that the differences are in the opposite direction to those actually observed.

‡ Calculation based on formula cited earlier and in (5). Males, $k = 0.73, f(T_a) = -17$. Females, $k = 0.48, f(T_a) = -7$. From each duration, 5 minutes are subtracted for warmup (2 minutes) and some E synthesis (3 minutes). Were the steps non-overlapping, the beginning would be when synthesis of E had been completed.

Interval Temperature

The relationship among pretreatment, interval temperature, and response to subsequent treatment is shown in Tables VIII and IX. For a given interval duration, the longer the pretreatment, the lower the maximal interval temperature. This supports the idea that higher temperatures favor the E-F conversion and lower temperatures favor the E-C″ conversion. When E (better D + E) is limiting, interval temperature becomes critical. This is another relationship emanating from the existence of a branch point, in this case where E can be converted to F or to C″.

DISCUSSION

From Fig. 3 it is seen that the sequence ABDE is common to the pathways of crossvein deformation and of high temperature adaptation. In addition, a large number of responses to experimental treatment at various temperatures can be related to one scheme.

TABLE VIII

EFFECT OF PRETREATMENT DURATION AND
INTERVAL TEMPERATURE ON PROTECTION

Pretreatment		Interval		Treatment		Ratings	
Temper-ature	Dura-tion	Temper-ature	Duration	Temper-ature	Duration	Males	Females
°C	min.		min.		min.		
40.5	1	39.5	10	41.5	20	7.9	10.0
40.5	2	39.5	10	41.5	20	10.2	10.0
40.5	5	39.5	10	41.5	20	11.5*	11.0*
40.5	1	38.5	10	41.5	20	0.1	1.2
40.5	2	38.5	10	41.5	20	2.4	3.9
40.5	5	38.5	10	41.5	20	10.0*	10.0*
40.5	5	38.0	10	41.5	20	—	9.0*
40.5	5	37.0	10	41.5	20	9.6	10.0
40.5	5	36.0	10	41.5	20	8.8	7.0
40.5	5	35.0	10	41.5	20	3.0	3.0
40.5	5	34.0	10	41.5	20	1.6	3.9
40.5	5	30.0	10	41.5	20	2.3	1.5
40.5	5	26.0	10	41.5	20	2.6	5.6
40.5	1	23.0	10	40.5	35	0.0	0.0
40.5	2	23.0	10	40.5	35	0.0	0.0
40.5	4	23.0	10	40.5	35	0.0	0.0

All treatments are sufficient to cause extreme crossvein defects in the absence of pretreatments and intervals. Low ratings indicate protection by pretreatment + interval.
* Mostly dead.

The interpretation of the results presented previously (5) must now be modified, since it was suggested at that time that a single change, the inactivation of a protein, underlay the observed events. The present scheme is more complex, involving 9 states (*vs.* 2) and 8 changes (*vs.* 1). Two major modifications must now follow. First, one cannot extrapolate the observed duration-response curves back to the beginning. However, recent data and calculations (*e.g.* slope in Fig. 2) indicate that a linear extrapolation of G formation can be made back to within 5 minutes of the beginning of a single treatment. It would thus appear that the absolute values of the y-intercepts are incorrect, but not exceedingly so; and that the relative values are not far off.

The over-all Q_1 is now seen to be composite. It still has pragmatic value, but

it cannot support conclusions as to the nature of underlying reactions. However, the individual Q_1's do still support the hypothesis of tertiary structure change in protein. They are in the middle of the expected range, where 2.3

TABLE IX

DEPENDENCE OF PROTECTION
OF INTERVAL TEMPERATURE

A: Treatment, 37 min. at 40.5°

Time	Sex	Interval temperature, °C							
		35.5	35.0	34.5	34.0	33.5	33.0	32.5	32.0
min.									
5	Males	0.0	0.1	0.0	0.0				
	Females	0.3	0.6	0.0	0.0				
8	Males	10.0	7.4	6.3	2.9				
	Females	9.6	8.5	7.0	2.5				
10	Males	d	d	7.8d	6.9	5.2	7.1	2.9	2.8
	Females	d	d	4.5d	5.7	5.3	6.0	3.2	2.4
12	Males				d	d	9.0d	6.8	8.5
	Females				d	d	7.6d	7.8	7.2
14	Males				d	d	d	11.3d	d
	Females				d	d	d	10.5d	d

B: Treatment, 25 min. at 40.5°

		34.0	33.5	33.0	32.5	32.0
10	Males	6.6	3.3	5.5	0.4	1.7
	Females	6.0	4.0	5.8	2.4	2.5
12	Males	8.3	8.7	8.2	5.0	6.3
	Females	9.1	7.6	8.3	6.4	6.1
14	Males	10.8	10.2	10.6	10.5	9.8
	Females	10.5	9.1	10.0	10.4	9.1

d = all or most dead.
Interval duration was always 1 hour.

The column header for the Pretreatment duration at 40.5° spans "Time" and "Sex".

was near the upper limit. The hypothesis thus explains all the observations described here, though of course, it is by no means proved.

There are, however, certain phenomena which cannot be explained by the present scheme. One, the effect of pupal aging temperature (T_a) on response, has already been attributed to the rate of synthesis of protein A (5). It remains to be seen whether C formation also plays a significant role in this effect.

Completely inexplicable in the present terms are the responses to temperatures in the 36.5° range and in the 37.5° range. Nothing from the previous description of these responses (5) or from the present experiments provides a basis for relating these other ways of making crossvein defects to the one under discussion. Indeed, nothing can yet be said about the mechanisms of these two phenomena.

Temperature Adaptation

It has long been clear that temperature adaptation mechanisms vary widely, even with respect to the level of organization at which they operate. Moreover, even in the present limited study there appear to be several variations on more than one theme. It is interesting that the present scheme involves first a rather transient adaptation, followed by two more permanent types. If adaptation were expensive, this scheme would serve the animal well. We don't know the relative efficiencies of the various functional states A, B, C, C', C'', D, E, and F, either with respect to crossvein synthesis or to their other duties. It would not be unreasonable to speculate, however, that added resistance to high temperatures may have to be bought at the price of decreased efficiency. It is unnecessary to carry this line of reasoning further, but the common occurrence of such a pattern would not be strange.

Changes in Tertiary Structure

Considering the biological importance of tertiary structure of proteins and the great deal of recorded information on many aspects of the subject, it is surprising that it enters so little into thinking about temperature adaptation and temperature effects in general. It is known that tertiary structure influences enzymes' kinetics, working conditions, sensitivity to inactivation, antigenic properties, and behavior in fractionation devices. It is known that tertiary structure of proteins can be changed by heat (with a characteristically high temperature coefficient) and by various chemical agents. Yet the synthesis, consideration, and application of this simple array of information seem all too uncommon in biology. Two cases of particular interest, however, are a similar scheme of alteration of the luciferase (3) and an experiment leading to the increase of resistance of gelatin to changes induced by high temperature, by prior exposures to less high temperature (7).

Sensitivity of the Posterior Crossvein

There are three possible reasons that the posterior crossvein is the first structure to respond to heat at this time: (a) The protein that is most heat-labile is found only in the posterior crossvein, or functions only there. (b) The conditions in the posterior crossvein particularly enhance heat effects on tertiary structure of this protein or proteins in general. (c) The difference between

amount needed and amount present of this protein is smallest here. Without
proof, the third hypothesis is favored for the following reason: no matter what
array of treatments is used to produce crossvein defects according to the present
method, extreme defects are accompanied by frequent killing, and 100 per cent
killing follows more drastic treatments. If the relevant protein were not also
involved in some *vital* process, it should be possible to dissociate extreme pheno-
copying from death. It is, of course, possible to protect against crossvein de-
fects and thus defer any visible change in the veins to a time past that of the
onset of death. However, in such cases the treatment necessary to kill the pupae
is much more extreme, implying that the next most sensitive enzyme has now
been inactivated to a critical degree, and that it is involved in a vital process.

George Wald has frequently pointed out that although riboflavin is vital to
almost every cell in the body, a riboflavin deficiency is first manifest in the
form of cracks in the skin near the mouth and nose. This is just another sign
of differentiation; the reserve amount of riboflavin and the ability of the af-
fected cells to maintain their supply must be among the lowest in the body. A
similar state of affairs is here suggested for the protein under discussion. It
may be important in various places in the pupa, but as it is inactivated, the
pinch is felt first in the crossvein.

It is interesting to see how a rather general environmental factor, high tem-
perature, has a strong and specific effect on the posterior crossveins over a
long period of time. The protection effect can be induced at least as early as
the time of puparium formation, and crossvein defects can be produced from
18 to 27 hours after puparium formation. In contrast, visible morphogenesis
of the posterior crossvein is a much shorter process (12) and covers only a small
part of this period of sensitivity. So although many cases are known in which
sensitivity coincides with morphogenesis, this relationship is not universal.
And it seems likely that, with the use of certain kinds of teratogenic agents,
including heat, more examples will be found of the specific prevention of nor-
mal morphogenesis by the early alteration of proteins with an ultimate role in
such morphogenesis. In fact, numerous cases are known which would fit such
a scheme. For example, heat shock administered to larvae can result in ab-
normal bristles in the adult; this is one of many phenocopies long known and
catalogued by Goldschmidt (see bibliography in reference 1). Although *anlage*
at the cellular level may be involved, it would seem sounder epigenetically to
implicate a protein as the mediator of the effect. It would then, of course,
follow that some proteins remain intact and function in quite different con-
texts during insect development, a possibility that can be tested.

Related Findings

Certain other observations are of some interest here; they will be published
in detail in a forthcoming paper (6). First, it has been found that after partial

protection at various temperatures between 32 and 38° one obtains duration-response curves at 40.5° with reduced slopes, as would be expected if some of the protein were shielded from further alteration. The same is true on the descending limb of the protectability curve, namely when E is being converted to F. In this case, with a suitable interval between pretreatment at 40.5° and treatment at 40.5°, increasing the pretreatment length (and, therefore, reducing the amount of E) causes a progressive duration-response slope increase until the unprotected values are reached.

It is also noteworthy that the valley in the age-response curve (5) seems to be tied to the D to E step. A good response is obtained here if one begins with making E at 42.5° or higher. Complete treatments at these temperatures are unsatisfactory because death generally precedes extensive crossvein defects.

Future Course of the Investigation

It is clear that more direct evidence on the nature of the changes must be sought. Attempts using histochemical techniques and protein fractionation are now under way.

REFERENCES

1. GOLDSCHMIDT, R. B., Theoretical Genetics. Berkeley, University of California Press, 1955.
2. GORDON, C., SPURWAY, H., and STREET, P. A. R., An analysis of three wild populations of *Drosophila subobscura*, J. Genetics, 1939, **38**, 37.
3. JOHNSON, F. H., EYRING, H., and POLISSAR, M. J., The Kinetic Basis of Molecular Biology, New York, John Wiley & Sons Inc., 1954.
4. MILKMAN, R. D., The genetic basis of natural variation. IV. On the natural distribution of *cve* polygenes of *Drosophila melanogaster*, Genetics, 1962, **47**, 261.
5. MILKMAN, R. D., Temperature effects on day old *Drosophila* pupae, J. Gen. Physiol., 1962, **45**, 777.
6. MILKMAN, R. D., Further studies on temperature effects on *Drosophila*, data to be published.
7. PRECHT, H., CHRISTOPHERSEN, J., and HENSEL, H., Temperatur und Leben, Berlin, Springer Verlag, 1955.
8. PREVOSTI, A., Variabilidad genica en una poblacion natural de *Drosophila subobscura. Genetica Iberica*, 1952, **4**, 95. Translation by Roger Milkman available on request.
9. PROSSER, C. L., and BROWN, F. A., Comparative Animal Physiology, Philadelphia, W. B. Saunders Company, 1961.

10. Timofeeff-Ressovsky, N. W., Über den Einfluss des Genotypischen Milieus und der Aussenbedingungen auf die Realisation des Genotyps, *Nachr. Ges. Wissensch. Göttingen, Math.- Physik. Kl., Biol.*, n. f., 1934, 1, 53–104. Translation by Roger Milkman available on request.

11. Waddington, C. H., Genetic assimilation, Advances Genetics, 1961, 10, 257.

12. Waddington, C. H., The genetic control of wing development in *Drosophila*, *J. Genetics*, 1940, 41, 75.