DELAYED RECOVERY FROM CARBON DIOXIDE IN *DROSOPHILA MELANOGASTER*^{1,2}

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THE phenomenon of carbon dioxide sensitivity in *Drosophila* has been known since 1937 when L'HÉRITIER and TEISSIER discovered it in a laboratory strain of *D. melanogaster*. Rather than awakening from CO₂ narcosis within 15 minutes as flies of resistant strains do, sensitive individuals are killed by the action of the gas. The sensitivity is readily inherited maternally, while the transmission through males is possible but much less efficient (L'HÉRITIER 1948). L'HÉRITIER and HUGON DE SEOUX (1947) reported the infectious nature of the CO₂-sensitivity agent, and the agent has been called virus *sigma* (σ) since 1954. Stabilized and nonstabilized CO₂-sensitive strains have been described (BRUN and SIGOT 1955). Nonstabilized females produce both sensitive and resistant progeny, but females of the stabilized state always produce sensitives, so stabilized stocks are 100 percent sensitive. Many aspects of *D. melanogaster* CO₂ sensitivity have been reviewed by L'HÉRITIER (1948, 1951, 1958, 1962). A similar phenomenon is now known to exist in several species of *Drosophila* in widely separated areas (e.g. WILLIAMSON 1961).

This paper concerns a study of a *D. melanogaster* strain developed by the senior author from a female collected in Pittsburg, Texas. Although first thought to possess ordinary CO₂ sensitivity, the strain has since been shown to be very different from CO₂-sensitive strains. When members of the strain were first tested with CO₂ some failed to awaken within 15 minutes after exposure. However, unlike CO₂-sensitive flies most of these individuals recovered after longer periods of time, so that it was possible to breed flies displaying slow recovery. Following such selection, the strain originally designated No. 199 (McCRADY 1960, 1961), now called the Texas Delayed Recovery strain (TDR), was established. Six substrains have been developed, each from a single female; they are designated TDR-1 to TDR-6. Combination of third-chromosome genes *claret* (*ca*) and *scarlet* (*st*) with another substrain of these flies has led to the production of a delayed-recovery strain with easily recognizable bright orange eyes. This strain is called strain TDR-orange.

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EXPERIMENTS AND RESULTS

The nature of the delayed-recovery response:

Delayed-recovery flies were regularly kept at a temperature of 18 to 20°C. The apparatus and procedures used in making CO₂ tests were similar to those described by PLUS (1954). Flies were exposed in small glass (later, polyethylene) tubes connected in a series with rubber tubing. The tubes were placed in a water bath for standard CO₂ testing at 13 to 14°C. In these tests pure CO₂ was used for 15 minute exposures at one atmosphere of pressure. Individuals recovering in 15 minutes were considered resistant. The terms recovered and nonrecovered will be used to designate the activity of delayed-recovery flies at specific times after CO₂ exposure. The criterion for recovery was the ability of tested flies to stand and move about.

Tested delayed-recovery flies react like CO₂-sensitives during approximately the first 15 minutes of the recovery period. The wings of many are extended, and except for an occasional resistant fly, little movement is observed during this time. Uncoordinated leg and wing movements may begin after 20 or 30 minutes. The movements usually increase and within an hour a few individuals may be able to right themselves. Movements sometimes continue for 18 to 24 hours before recovery takes place, but most often recovery occurs within a 4- or 5-hour period or not at all. Although movements of nonrecovered flies may even continue beyond 24 hours, such individuals seldom survive.

After testing, delayed-recovery flies are capable of feeding and, in some instances, egg laying; but they often remain weak, having slow and limited movements. Moreover, some flies capable of standing and walking for a time die within the next few hours. Because of the varying degrees of recovery, classification of delayed-recovery individuals into recovered and nonrecovered groups after CO₂ testing is often difficult and necessarily somewhat arbitrary.

Although all TDR flies exhibit characteristic recovery movements after testing, the percentage of survivors was found, especially in the early tests, to vary greatly between culture bottles of the same test as well as between tests. In one CO₂ test, for example, 750 flies were dumped from six culture bottles and tested simultaneously in separate tubes. The percentage which had recovered 18 to 24 hours after testing varied from 52 to 90 percent depending on the bottle from which the flies had been taken. It was later found that some of this variation could be eliminated by preventing excessive larval crowding and by not testing flies until they were at least three or four days old. When such steps were taken, a recovery of about 70 percent was commonly found following a standard test (see Tables 3 and 4).

Hereditary basis of delayed-recovery: A series of crosses has been made for the purpose of determining the mode of inheritance of delayed recovery. Strain TDR and resistant (Oregon) flies were first crossed reciprocally. Some of the F₁ progeny were CO₂ tested and some were either backcrossed to Oregon flies or mated to other F₁ progeny of the same cross. The offspring of the F₁ crosses and backcrosses were also subjected to CO₂ under standard testing conditions. The results are given in Table 1.

Although only 3.7 percent of the total F₁ progeny (a and b) recovered in 15

minutes, 98.3 percent had recovered within an hour after testing. Only a few uncoordinated movements occurred before these flies were able to right themselves, and the recovered flies did not appear weakened by the test. This characteristic recovery behavior of F₁ flies was shown for an average of about half (45.3 percent) of the backcross progeny (c-f). The other approximate half (54.1 percent) of the progeny were resistant to CO₂, while only 0.6 percent required more than an hour for recovery. Three major categories of F₂ progeny can be noted (g and h). One group (25.1 percent), reacting like resistant flies, recovered within 15 minutes. A second group, the largest (56.3 percent), recovered between 15 minutes and 1 hour after testing, thus behaving like most of the F₁ progeny. Members of the third group (18.6 percent) required more than an hour for recovery and displayed the uncoordinated recovery movements of strain TDR.

The CO₂ recovery behavior for progeny of the crosses shown in Table 1 is reasonably consistent with what would be expected if the delayed-recovery phenomenon is determined primarily by a single semidominant gene. Designating this gene, *Dly*, individuals homozygous for *Dly* would be delayed-recovery flies, not generally recovering within 1 hour after testing. As determined by the recovery behavior of the F₁, the heterozygotes would be represented, for the most part, by individuals awakening between 15 minutes and 1 hour after testing. Being resistant, flies not carrying *Dly* would be expected to awaken within 15 minutes after testing. Based on the hereditary determination of delayed-recovery by a semidominant gene, expected recovery percentages of backcross and F₂ progeny were computed from what had been determined about the recovery times of individuals having the genotypes of their parents. Essentially, these expected values reflect the basic expected 1:1 ratio for backcross progeny and the 1:2:1 ratio for the F₂ progeny, but the fact that heterozygotes (a and b) did not always awaken within the interval of 15 minutes to 1 hour is taken into account. The expected values are given in parentheses beneath the observed recovery percentages for each time interval. Usually the correspondence between observed and expected percentages is fairly good, though some of the differences are larger than one might expect. In addition to chance variation, the deviations from the expected percentages were probably due primarily to judgment errors as to the time of recovery for some individuals and to variation in gene expression. The existence of *Dly* is no longer in doubt, because it has been possible, as discussed below, to locate the factor on a chromosome.

To establish the specific chromosomal association of the hereditary determiner of delayed-recovery, use was made of the well known second and third chromosome balanced lethal stock *Pm/Cy; Sb/D* (*Pm*, Plum eyes; *Cy*, Curly wings; *Sb*, Stubble bristles; *D*, Dichaete wings). F₁ "Pm Sb" and "Cy Sb" males from the cross of *Pm/Cy; Sb/D* females to TDR males were outcrossed to Oregon females. The offspring were classified as to Curly, Plum, and Stubble characteristics and then subjected to CO₂. Checking to determine recovery behavior showed that none of the combined 622 "Sb", "Cy Sb", and "Pm Sb" offspring of these crosses expressed any delayed-recovery symptoms; they all recovered within 15 minutes after testing. Conversely, of the combined "Cy" and "Pm" offspring, only 5.5

TABLE 1

CO₂ testing of progeny from crosses involving strain TDR and Oregon

Cross	Offspring tested	Percent recovered		Percent not recovered by 1 hr
		0-15 min	15-60 min	
a. Oregon ♀ × TDR ♂	1308	6.0	92.6	1.4
b. TDR ♀ × Oregon ♂	1308	1.5	96.6	1.9
c. Oregon ♀ × F ₁ ♂ (Oregon ♀ × TDR ♂)	956	56.0 (53.0)	43.6 (46.3)	0.4 (0.7)
d. Oregon ♀ × F ₁ ♂ (TDR ♀ × Oregon ♂)	1166	53.0 (50.7)	45.8 (48.3)	1.2 (1.0)
e. F ₁ ♀ (Oregon ♀ × TDR ♂) × Oregon ♂	1587	50.4 (53.0)	48.9 (46.3)	0.7 (0.7)
f. F ₁ ♀ (TDR ♀ × Oregon ♂) × Oregon ♂	1224	58.4 (50.7)	41.5 (48.3)	0.1 (1.0)
g. F ₁ ♀ × F ₁ ♂ (Oregon ♀ × TDR ♂)	2371	25.7 (28.0)	56.6 (46.3)	17.7 (25.7)
h. F ₁ ♀ × F ₁ ♂ (TDR ♀ × Oregon ♂)	664	23.0 (25.7)	55.4 (48.3)	21.6 (26.0)

percent (12/219) were able to walk within 15 minutes; usually these individuals had recovered within an hour. This is clearly indicative of the heterozygous expression of *Dly*. Likewise, heterozygous-type delayed recovery was shown for 99 percent (200/202) of the otherwise wild-type progeny of the cross. Since none of the "Sb" offspring expressed delayed-recovery whereas nearly all of the progeny in the "non-Sb" category did, it was concluded that *Dly* must be located on the same chromosome as *Sb*, the third.

Testcrosses utilizing third-chromosome markers were next made for the allocation of *Dly* to a specific third-chromosomal locus. TDR females were mated to males homozygous for the recessive genes *th* (thread aristae), *st* (scarlet eyes), *cu* (curled wings), *sr* (striped thorax), *e* (ebony body color), and *ca* (claret eyes). F₁ females were mated individually to the multiple mutant males at 25°C. After six or seven days the parents were removed, and the crosses were placed at 18°C, because it had been shown, as will be discussed, that high temperatures cause a diminishing of the delayed-recovery response to CO₂, especially if late developmental stages are exposed. The backcross progenies were classified as to the expression of the marker genes and then subjected to CO₂ for the detection of heterozygous-type delayed recovery. The recombination values obtained showed *Dly* to be near the *cu* locus at 50.0.

A 4-point testcross was then made which used the *st*, *cu*, and *sr* markers. A total of 1362 offspring were scored. The recombination values in this case showed *Dly* to be 2.7 units to the right of *cu*. With these data the map distances obtained for the known markers corresponded well with those of the standard map, so 52.7 has been accepted as a good estimate of the position of *Dly*.

Evidence for an artificially transmissible agent in strain TDR: After *Dly* was

found to be the apparent hereditary determiner of delayed recovery, the results of certain injections have indicated the presence of a σ -like infectious agent in members of the TDR strain. Several series of injections of extract from TDR flies into resistant flies of various strains have been made.

Preparation of the extracts was accomplished by crushing, in ice, 100 to 350 individuals in 1 ml of Bodenstein's solution (1000 ml distilled water, 7.5 g NaCl, 0.35 g KCl, 0.21 g CaCl₂). The crude extract was transferred to a sterilized centrifuge tube for 15 min refrigerated centrifugation at $1215 \times g$. Using a Swinny adaptor, the supernatant was filtered through an asbestos or Millipore filter (450 m μ) in some experiments, while in others the decanted supernatant was used without further manipulation. For injections a glass microinjection needle was inserted into the abdomen of etherized flies generally less than one week old. Injection pressure was supplied by pressing a 3-inch rubber bulb connected with tubing to the needle holder. Transfers of injected flies to fresh food vials were made every four to eight days, and the flies were tested with CO₂ at various time intervals to determine their response.

The results of the early sets of injections, while not conclusive, suggested further trials. In the most successful series, 159 Oregon and 50 resistant flies of a strain called the Pittsburg strain were injected. (The Pittsburg strain was derived from an isolated female collected in Pittsburg, Texas.) The extract was made by crushing 150 TDR flies in 1 ml of BODENSTEIN'S solution and centrifuging without filtration. Tested 33 days after injection, 18 of the 21 surviving Oregon and five of the seven surviving Pittsburg flies were killed by the CO₂ exposure. Offspring were recovered from the vials in which the injected individuals had been kept just prior to testing. Five of 19 Oregon and two of 11 Pittsburg strain offspring were CO₂-sensitive. Part of the F₁ were not tested, and these individuals served as a basis for obtaining an F₂, which, also being partly sensitive, was used to establish four neostabilized strains. Three of these were from the originally injected Oregon strain and were designated TDR-Oregon. Only one of these strains was kept. The other strain was developed from the injected Pittsburg strain and was, accordingly, called TDR-Pittsburg.

Further CO₂ testing has demonstrated that the response of the two remaining strains resembles true CO₂ sensitivity in the sense that nearly all individuals are killed by CO₂. Of 382 TDR-Pittsburg flies initially tested, only seven survived, and for TDR-Oregon flies survival resulted for only two of 314. Strain TDR-Oregon has since been used for CO₂ testing experiments (e.g. Table 4b) and as a source of extract to induce sensitivity by injection. It is now virtually 100 percent sensitive and is considered as a stabilized CO₂-sensitive strain.

The maternal transmission of the hereditary determiner of CO₂ sensitivity in the TDR-Oregon strain and the TDR-Pittsburg strain has been demonstrated by the results of reciprocal outcrosses involving these strains as shown in Table 2. The efficiency of maternal inheritance of sensitivity is evident, while the limited paternal transmission is in keeping with the expected transmission through a low valency male.

Although several injection series intended to confirm the presence of a CO₂-sensitivity producing agent in TDR flies led to only negative or equivocal results, one recent injection series was more successful. In this series, 300 Oregon and 90

TABLE 2

CO₂ exposure at 14°C for 15 minutes of outcross progeny of induced sensitive strains

Cross	Number	Offspring	
		Percent sensitive	Percent resistant
Oregon ♀ × TDR-Oregon ♂	555	5	95
Oregon ♀ × TDR-Pittsburg ♂	432	6	94
TDR-Oregon ♀ × Oregon ♂	534	99	1
TDR-Pittsburg ♀ × Oregon ♂	388	97	3

flies of a CO₂ resistant strain called the Lincoln strain were injected. (The Lincoln strain was started from a female collected in Lincoln, Nebraska.) For the extract, 350 strain TDR-orange flies were crushed in 1 ml of BODENSTEIN's solution and the material was filtered through a Millipore filter. CO₂ testing of the injected flies was first conducted 33 days after injection. Of the injected Oregon flies, 63 survived until this test, and ten of these had not recovered within 15 minutes. Seven of the ten recovered later, but three died. Only four Lincoln flies lived until the first CO₂ test, but two of these did not recover from it. Thirteen days after the first test 10 of 30 surviving Oregon flies failed to recover from a second test, and one of the two remaining Lincoln flies did not recover from this test. Some apparently sensitive progeny were collected from vials which had contained the injected individuals. Most of these developed from eggs which the injected flies had laid during the period approximately 10 to 20 days after injection. The response of these offspring to CO₂ testing was as follows:

Strain	n	Percent not recovered after		
		15 min	3 hr	7 hr
Oregon	831	14	9	10
Lincoln	242	12	10	17

(Some of the flies able to stand three hours after CO₂ exposure were unable to maintain this function and were thus recorded as nonrecovered at the 7-hour check.)

Sensitivity in the Lincoln strain has not been perpetuated, but some descendants of the injected Oregon flies continue to be killed by CO₂, while others survive only after exhibiting a delay in recovery. The condition has not yet been stabilized, so less than 100 percent of these descendants express an unusual response to CO₂. The nature of the response has not been studied further, and analysis has not yet been made of the hereditary basis of the condition. However, nonstabilized lines are being maintained for later study.

Factors influencing the delayed-recovery response: (1) *Length of CO₂ exposure.* Subjecting separate groups of 100 or 200 delayed-recovery flies to pure CO₂ for varying lengths of time (15 sec to 1 hr) has shown that recovery time and lethality increase as the duration of exposure is increased (Table 3). CO₂ exposures of longer than 15 seconds will kill CO₂-sensitive flies (L'HÉRITIER 1948), but no mortality occurred for delayed-recovery individuals exposed up to

TABLE 3

Exposure of strain TDR to CO₂ at 14°C for various periods of time

Length of test	Number	Percentage apparently recovered after									
		3 min	5 min	10 min	15 min	30 min	1 hr	2 hr	4 hr	8 hr	16 hr
15 sec	100	100	100	100	100	100	100	100	100	100	100
30 sec	100	56	100	100	100	100	100	100	100	100	100
1 min	100	43	100	100	100	100	100	100	100	100	100
2 min	100	0	36	77	89	95	100	100	100	100	100
4 min	100	0	0	3	6	33	81	100	100	100	100
8 min	100	0	0	0	0	2	61	92	93	93	93
15 min	200	0	0	0	0	0	7	45	61	72	71
30 min	200	0	0	0	0	0	5	18	29	40	48
60 min	200	0	0	0	0	0	0	11	23	29	40

4 minutes in this testing series. For each exposure time considerable individual variation in recovery time can be noted. Thus, for example, the first flies to awaken after a 15-minute CO₂ test recovered sooner than the last to awaken after a 4-minute exposure.

(2) *Temperature of CO₂ exposure:* A series of CO₂ exposures of Oregon, TDR-Oregon and TDR-1 flies has been conducted over a testing temperature range of 2 to 38°C with intervals of 2° (Table 4). The results of testing Oregon (resistant) flies at temperatures from 24° to 38° are shown in Table 4a. These resistant flies recovered within 15 minutes when exposed to CO₂ at 24° or below. However, when tested above 24°, recovery time was longer, and above 32° recovery was either very prolonged or did not occur.

Table 4b shows the results of testing TDR-Oregon (CO₂-sensitive) flies at temperatures from 14 to 38°C. Death resulted for all members of this strain tested at a temperature below 16°. When CO₂ exposures were made at 16° to 20°, less than 10 percent survived, but recovery at 22° was 58 percent, at 24°, 78 percent; and in the 26 to 32° range, 94 to 100 percent. A lack of expression of CO₂-sensitivity when testing at higher temperatures was expected, because L'HÉRITIER (1948) reported that sensitivity does not manifest itself with testing temperatures above 23°. Table 4b shows, however, that in the testing temperature range where recovery occurred, recovery time for sensitive TDR-Oregon flies increased directly as the testing temperature was increased. At temperatures above 28°C members of the strain never recovered in the first 15 minutes after testing. It was also noted that the behavior of these recovering flies was much like that involved in the recovery of strain TDR in that recovery was preceded by much uncoordinated movement. When the testing temperature for strain TDR-Oregon was above 32°, recovery of survivors was always much prolonged, and it occurred only 25 to 58 percent of the time. This slow recovery or lethality manifest at higher testing temperatures apparently has no relationship to maternally transmitted CO₂-sensitivity, since resistant individuals tested at above 26° reacted in a similar manner. Death in such cases, however, is not due to the

TABLE 4

Exposure of flies to CO₂ at various temperatures for 15 minutes

Temperature °C	Number	Percentage apparently recovered after							
		15 min	30 min	1 hr	2 hr	4 hr	8 hr	16 hr	32 hr
a. Oregon (CO ₂ -resistant)									
38	150	0	0	0	0	0	0	43	47
36	118	0	0	0	8	3	11	64	66
34	353	0	0	0	2	5	31	37	40
32	225	0	0	36	77	98	99	98	95
30	160	0	0	10	98	100	100	100	100
28	208	0	27	86	99	100	100	100	100
26	106	70	100	100	100	100	100	100	100
24	115	100	100	100	100	100	100	100	100
b. Strain TDR-Oregon (CO ₂ -sensitive)									
38	89	0	0	0	0	0	0	10	25
36	147	0	0	0	0	0	2	54	58
34	277	0	0	0	1	1	24	53	54
32	175	0	0	19	69	94	99	99	97
30	132	0	0	14	99	100	100	100	100
28	202	0	15	73	91	96	96	96	96
26	205	28	70	86	94	94	94	94	94
24	106	32	63	72	73	78	78	78	78
22	175	23	47	55	57	58	58	58	58
20	288	0	3	7	9	9	9	9	9
18	153	0	0	2	9	10	9	6	6
16	111	4	5	9	9	8	8	8	8
14	146	0	0	0	0	0	0	0	0
c. Strain TDR-1									
38	77	0	0	0	0	0	0	0	0
36	83	0	0	0	0	0	0	2	6
34	185	0	0	0	0	1	2	6	8
32	150	0	0	0	0	1	1	5	11
30	175	0	0	0	1	1	6	6	12
28	167	0	0	0	4	8	17	12	12
26	101	0	0	0	7	18	19	19	19
24	137	0	0	0	3	11	12	16	17
22	213	0	0	0	2	8	16	20	20
20	259	0	0	1	37	49	58	56	56
18	219	0	0	6	32	33	69	58	58
16	263	0	0	1	18	53	70	74	74
14	211	0	0	4	48	64	72	71	71
12	167	2	5	14	63	74	74	74	74
10	102	8	10	26	63	90	92	92	92
8	89	6	26	81	95	99	99	99	99
6	176	39	88	95	100	100	100	100	100
4	76	83	100	100	100	100	100	100	100
2	126	78	100	100	100	100	100	100	100

effect of heat alone. Both sensitive and resistant flies exposed in air to temperatures of up to 38° for 15 minutes were not noticeably damaged.

The results of testing TDR-1 (delayed-recovery) flies at temperatures of 2° to 38°C are given in Table 4c. This strain is seen to have been much less severely influenced by CO₂ at low testing temperatures than TDR-Oregon. At 2 to 4° all of the TDR-1 flies tested recovered within 30 minutes, thus behaving much like resistant strains. The survival remained high (92 to 100 percent) and recovery time short in the 6 to 10° testing range. Testing at 12° to 20° resulted in a response much the same as that which had been found earlier at 14°, although recovery was somewhat higher and recovery time shorter at the lower end of the range. When tests were made at 22° a sharp drop in survival to 20 percent resulted as compared to 56 percent at 20°, and above 22° the survival percentages were even smaller. Recovery of survivors at these temperatures was greatly prolonged. Only occasional survivors, all of them extremely weak, occurred when testing was conducted above 30°.

The comparison of survival of delayed-recovery TDR-1 flies and CO₂-sensitive TDR-Oregon flies is shown more clearly in Figure 1. (The graph does not show the longer recovery period required at higher testing temperatures; only the total percentages of recovered individuals are indicated.) It is evident that when tested at temperatures of up to 30°C, the recovery curves for these two strains are essentially reversed due to the TDR-1 flies surviving to the greatest extent when tested near 0°, while survival of TDR-Oregon individuals was the highest at 30°. It is of interest to note that the greatest single decrease (36 percent) in survival for strain TDR-1 occurred when the testing temperature was raised from 20° to 22°, and this temperature increase also resulted in the greatest single increase (49 percent) in survival for strain TDR-Oregon. Tested above 30°, survival of both strains dropped from what it had been at that temperature, but while the strain TDR-1 recovery percentage dropped from 12 percent at 30° to zero at 38°, 25 percent of the TDR-Oregon flies recovered at 38° compared to 100 percent at 30°. The graph also shows that the survival of the resistant Oregon flies dropped sharply when the testing temperature was raised above 32° in a manner somewhat similar to what was found for the CO₂-sensitive TDR-Oregon strain.

FIGURE 1.—Total recovery by 32 hours of Oregon (broken line), TDR-Oregon (solid line) and TDR-1 (dotted line) from 15 minute CO₂ exposure.

(3) *The effects of nitrogen testing*: Preliminary tests with nitrogen have been made on members of strain TDR to determine if delayed recovery represents a general response to anoxia rather than a specific CO₂ effect. Ordinary CO₂-sensitive strains are not sensitive to nitrogen; they behave like resistant flies when exposed to the gas (L'HÉRITIER 1948).

Subjecting 100 TDR flies to one atmosphere of pure nitrogen at 15°C for 15 minutes resulted in neither lethality nor delayed recovery; all flies recovered in 15 minutes. The same result was obtained when 63 TDR flies were nitrogen tested at 22°. As expected, members of two CO₂-sensitive strains and two resistant strains also recovered within 15 minutes after a 15 minute nitrogen exposure. It is shown in Table 5 that nitrogen testing at 30° resulted in a delay in recovery of all strains tested, though to a lesser extent than was evident after CO₂ exposure at this temperature. (The Niobrara strain is a CO₂-sensitive strain established from a female collected in Niobrara State Park, Nebraska. The Oakland strain is a CO₂-resistant strain started from a female collected by DR. B. W. GEER at Oakland, California.) Since anoxia due to nitrogen exposure resulted in a similar response for resistant, CO₂-sensitive, and delayed-recovery strains, delayed recovery from CO₂ exposure of TDR flies must be more than an increased manifestation of anoxia compared to that found in strains not having gene *Dly*.

(4) *Heat curing of strain TDR*: When adult delayed-recovery flies were kept at 30°C for four or eight days, they behaved, when exposed to CO₂ under standard conditions, the same as individuals which had been kept at 18°. A somatic heat cure of adults was not accomplished. On the other hand, TDR flies allowed to develop at 30° did not show the characteristic delayed-recovery response to CO₂; 92 percent of 238 flies tested recovered within 15 minutes and by 45 minutes 100 percent recovery had occurred.

In a later study TDR flies were carried through as many as five generations at 30°C. Forty-nine individuals whose ancestors had been heat treated through three generations and 57 flies whose ancestors had been heat treated through five generations did not display delayed recovery when tested immediately after their removal from that temperature; they all recovered within 15 minutes. In contrast, when flies whose ancestors had been heat treated were removed (as adults) from the 30° incubator and placed at 19°, their offspring (32, 168, and 232 flies respectively of 3rd, 4th, and 5th generation ancestors kept at 30°) all

TABLE 5
Nitrogen exposure at 30°C for 15 minutes

Strain	Number	Percentage recovered after		
		15 min	30 min	60 min
TDR	85	5	81	95
TDR-Oregon	35	34	100	100
Niobrara	68	3	53	91
Oregon	16	56	100	100
Oakland	113	4	86	100

displayed delayed recovery. It is thus apparent that the heat induced cure was only somatic, the germinal cells not being affected. This constitutes a difference from what has been found for ordinary CO₂-sensitive strains for which varying germinal heat cures have been reported (L'HÉRTIER 1958; DE LESTRANGE 1963).

After developmental somatic heat cure was established, emerging strain TDR adults showed little reversion to sensitivity even though CO₂ testing was postponed for extended periods. When 38 adults reared at 30°C through three generations and 108 reared at this temperature for five generations were placed at 19° for two weeks before being CO₂ tested, 87 percent of the former and 97 percent of the latter recovered within 15 minutes. The somatic heat cure was, therefore, considered to be "permanent" rather than "temporary". L'HÉRTIER and SIGOT (1946) reported that CO₂-sensitivity which is somatically cured by heat treatment is characteristically only "temporary", because a reversion to sensitivity occurs soon after removal to 19°C.

In a study to determine the stage of development during which heat treatment is most effective, egg, larval and pupal stages were subjected to 30°C heat treatment for varying lengths of time (Table 6). After treatment of eggs, an average of 56 percent of the emerging adults recovered from a standard CO₂ test within an hour, and all but one percent recovered within 5 hours. These recovery times point to some lessening of delayed recovery compared to untreated flies (see Tables 3 and 4). Treatment of first instar larvae had no appreciable curative effect in terms of diminishing recovery time (an average of only 10 percent recovery within 1 hour after testing), but 92 percent survived. Leaving third instar larvae at 30° for 72 hours resulted in slightly more recovery after 1 hour (17 percent) for CO₂ tested emerging adults than expected for untreated flies, but otherwise heat exposure of second and third instar larvae had no detectable influence. The results of the pupal heat treatment clearly show its curative effect. An average of 93 percent of the adults emerging from these pupae recovered within 30 minutes after CO₂ testing, and 100 percent recovery occurred by 1 hour.

The heat treatment periods often extended from one developmental stage to the next. Eggs had hatched into first instar larvae by the time the treatment was completed, but since larval heat treatment was not found to be effective in curing, this would not be expected to influence the results. Second instar larvae which were left at 30°C for 72 hours had pupated before they were returned to 18°, but the data show that this did not affect a cure. On the other hand, the slight effect brought about by heat treatment of third instar larvae for 72 hours may have been due to these larvae having pupated long before the treatment period ended. The especially effective cure resulting from 72 hour pupal treatment may have been due in part to the emergence of adult flies while still at 30°. However, 16 flies obtained from pupae which were near eclosion when placed at 30° ("old pupae") still displayed partial delayed recovery (only 50 percent recovery after 1 hour) even though they all emerged as adults during the treatment. Thus, complete heat curing apparently required heat treatment of earlier pupae.

TABLE 6

Recovery of strain TDR adults from 15 minute CO₂ exposure at 14°C after 30°C developmental heat treatments of varying duration

Heat treatment beginning with:	Treatment period (hours)	Number	Percentage apparently recovered after				
			15 min	30 min	1 hr	2 hr	5 hr
Eggs	24	10	0	0	60	80	100
Eggs	36	38	1	51	64	96	98
Eggs	48	43	0	30	49	100	100
Larvae							
1st instar	36	9	0	0	11	44	100
1st instar	48	28	0	11	14	54	86
1st instar	72	13	0	0	0	77	100
2nd instar	36	20	0	0	0	15	75
2nd instar	48	45	0	0	2	62	68
2nd instar	72	72	0	0	6	83	84
3rd instar	36	52	0	0	2	57	80
3rd instar	48	40	0	0	5	42	80
3rd instar	72	64	2	6	17	77	76
Pupae	24	11	0	100	100	100	100
Pupae	36	16	6	88	100	100	100
Pupae	48	22	35	95	100	100	100
Pupae	72	12	67	100	100	100	100
Old pupae	48	16	13	13	50	100	100

(5) *Studies involving two CO₂ exposures:* When members of the TDR strain were subjected to CO₂ for a second time they acted entirely differently than after their initial exposure. Following such second tests, most individuals were active by 15 minutes after their return to air. Furthermore, a second CO₂ dose was often beneficial to individuals which had apparently been injured by the first exposure to the gas. Even when the first CO₂ exposure was short, it was shown to greatly modify the results of a CO₂ test performed later. In one testing series the exposure times of the first tests were 15 sec, 30 sec, 1 min, 2 min, 4 min, and 8 min. Fifty flies were exposed in each test. After varying degrees of delayed recovery, all of these flies survived except for six of those which had been tested for 8 minutes. Recovery time from a standard 15 minute test performed on the flies one week later was reduced to 20 minutes or less in all cases.

Other data have indicated that the amount of time intervening between two CO₂ exposures may make some difference in the response to the second exposure, but the second response was always much less severe than the first. Strain TDR flies were subjected to CO₂ for 2 minutes. Delayed recovery was exhibited, but although many of the flies were still weak after an hour, they could stand by this time. These individuals eventually recovered fully. They were CO₂ tested at intervals, and the percent of recovery after 15 minutes was checked. The results are given in Table 7. It is seen that a first exposure to CO₂ influenced the results of the second throughout almost the entire life span of the flies.

TABLE 7

CO₂ testing at 14°C for 15 minutes of TDR flies previously exposed for two minutes

Number	Interval (days)	Percent recovered in 15 minutes
100	2	100
85	9	100
100	26	75
76	32	77
43	38	81

"Subliminal" treatments of CO₂ have been found to adapt regular CO₂-sensitive flies to otherwise lethal exposures (L'HÉRITIER 1948; TATTERSFIELD and KERRIGE 1955). This adaptation is of short duration, however, apparently lasting only a matter of minutes. As is true for CO₂ adaptation of the TDR strain, the mechanism of the adaptation of CO₂-sensitives is not known.

Strain TDR flies subjected to CO₂ two or more times produced offspring which were as susceptible to CO₂ as were their parents when exposed for the first time. Thus, CO₂ treatment seems to influence only the somatic cells. It has also been shown that parents subjected to CO₂ for 15 minutes produced progeny which responded to CO₂ in the same manner as those progeny whose parents were exposed for less than 2 minutes; they all underwent characteristic delayed-recovery responses.

DISCUSSION

Delayed recovery of strain TDR represents an interesting departure from the usual response of *D. melanogaster* to carbon dioxide exposure. Homozygosity for the third-chromosomal gene *Dly* has been shown to be primarily responsible for the slow and uncertain recovery exhibited by members of strain TDR to a "standard" 15 minute CO₂ exposure at 14°C. Outcrosses to resistant Oregon flies (Table 1) have demonstrated that the gene acts as a semidominant, causing an intermediate effect in the heterozygous state. Delayed recovery is a good term for the phenomenon, because if recovery from CO₂ occurs it is nearly always delayed regardless of the length of exposure or the temperature at which exposure is made (Tables 3 and 4). The condition appears to be unique, and it should not be thought of as a less severe form of CO₂-sensitivity; after CO₂ testing at temperatures of 22 to 32°C, members of the delayed-recovery strains are usually killed, whereas sensitive flies tested at these temperatures are not (Table 4).

Little has been conclusively demonstrated concerning the mode of action of *Dly*. Its action may take place mainly during the pupal state, since placing pupae at 30°C for varying lengths of time consistently results in the production of adults which do not exhibit delayed-recovery (Table 6). Heat treatments of eggs resulted in only a partial cure of delayed-recovery symptoms, and larval heat treatment was generally ineffective. The fact that in a given generation a second dose of CO₂ elicits a response similar to that of resistant flies is probably signifi-

cant in understanding *Dly* action, but, as is also true of the somatic heat cure, nothing is presently known as to how this cure is brought about.

As shown by nitrogen testing, whatever is involved physiologically in delayed-recovery, the phenomenon does not result from anoxic conditions alone. Tests with other gases will be necessary, however, before the condition can be considered as a specific manifestation of CO₂ exposure.

Strain TDR fly extracts have been shown capable of occasionally producing a type of CO₂-sensitivity when injected into resistant strains. The sensitivity resulting from one series of such injections has been shown to be identical, or nearly identical, to σ -produced sensitivity. These results show that viral (or viral-like) infectious particles similar to σ must exist in some, if not all, TDR flies. Because of the difficulty usually encountered in inducing CO₂-sensitivity with strain TDR extracts, it has not been possible to assay the titer of the infectious units in TDR flies by determination of the incubation period. But the low total percentage of induced sensitivity which generally occurred even after long incubation periods points to an unusually low titer. It is not presently known if the σ -like agent is important in producing delayed recovery, and knowledge is also incomplete as to what, if any, relationship exists between *Dly* and the production of, and/or the interaction with, this agent. Some possibilities will be considered briefly based on the limited information now available.

The results of the reciprocal crosses shown in Table 1 give no apparent evidence of maternal inheritance being involved in the transmission of delayed recovery. Therefore, when injection experiments later revealed the apparent presence of a cytoplasmic σ -like agent in the TDR strain, it was thought that the agent necessarily represented a distinct maternally inherited entity which was not involved in the production of the delayed-recovery phenotype. This idea is still to be considered as a distinct possibility. In this hypothesis the mechanism of somatic heat cure of delayed-recovery symptoms in strain TDR would be considered as one by which the gene action is modified directly rather than one by which the production of the infectious cytoplasmic agent is inhibited. That heat shock at an appropriate developmental stage could interrupt *Dly* action is not unlikely. Such temperature sensitive periods of specific gene action in *Drosophila* have been found by several investigators. This work has been summarized by GOLDSCHMIDT (1938) and ROEDER (1953).

The hypothesis which considers *Dly* and the cytoplasmic agent in strain TDR as distinct in origin and function is given added support at the present time because, as evidenced especially by their entirely different responses to CO₂ at different testing temperatures, delayed recovery and CO₂-sensitivity seem to represent two different phenomena. Since the cytoplasmic agent is, in this hypothesis, considered to be maternally inherited like σ , it could not be thought of as being important in the production of the delayed-recovery phenotype; delayed recovery is not maternally inherited. Maternal inheritance of the agent in the TDR strain has not, however, been proven. In addition, there are several possible objections to the hypothesis in question. First, it seems unlikely, on the basis of chance, that an individual taken from nature would carry both *Dly* and a

separate CO₂-sensitivity producing agent. The meager information which is available indicates a low incidence of both delayed recovery and CO₂-sensitivity. To our knowledge there have been no other reports of delayed recovery, but there has not been a systematic survey to find it in other wild-caught flies. WILLIAMSON (1959) found that 102 of 6,301 (1.6 percent) of the *D. melanogaster* from Lincoln, Nebraska which he tested were CO₂-sensitive; no further information on the incidence of the phenomenon in the United States can be given. Perhaps a more serious objection is that if mature CO₂-sensitivity producing particles are present in some or possibly all members of strain TDR, it would be expected that at least occasional manifestations in response to CO₂ testing would be detectable in these members. Since the delayed-recovery phenotype is manifested by members of the TDR strain, it seems likely, that the cytoplasmic agent *per se* may in some way be involved in the production of this phenotype. A third possible objection to the hypothesis is the apparently consistently low titer of the infectious σ -like agent in strain TDR. This indicates some controlling action on the production of the agent. The control could well be a manifestation of *Dly* action.

In the two alternative hypotheses to be presented, it will be presumed that *Dly* and the infectious agent in the TDR strain are in some way functionally related. The first alternative possibility is that *Dly* is responsible for controlling the maturation of the infectious agent. If such a gene controlled influence preventing the production of a high titer of the σ -like agent is in operation in strain TDR, it would be probable that the strain would also be partially resistant to the rapid multiplication of introduced infectious σ . Injections have been started to determine whether such a partial immunity exists, and results to date suggest that it does. These results will be reported later.

In addition to controlling the maturation of the infectious agent, *Dly* must, in this hypothesis, also be primarily responsible for the delayed-recovery response. This is true because, as in the first hypothesis, the agent is considered to be inherited maternally, so the known mode of inheritance of delayed recovery excludes the possibility of this agent playing an important part in the production of the phenomenon. It should also be noted that in this hypothesis as in the first no explanation is given as to the origin of the infectious agent.

Perhaps the most interesting possibility is that *Dly* is functionally similar to the proposed hereditary determiner of stabilized CO₂-sensitivity as well as of the states called "rho" and "ultra-rho." L'HÉRITIER (1962), based in part on the work of BRUN (1963), has proposed that females of these strains contain a noninfectious form of σ called "genetic virus," which is considered responsible for the genetic continuity of σ . In CO₂-sensitive strains "genetic virus" is also thought to be responsible for the production of infectious σ . Rho flies, derived from the stabilized sensitive state, only rarely produce infectious virus and ultra-rho flies, derived from rho, never do. "Genetic virus" has been shown not to be chromosomally integrated, but its other similarities to temperate phage (prophage) of lysogenic bacteria have been stressed by L'HÉRITIER (1962). An important similarity is that of immunity. Lysogenic bacteria are immune to superinfection with

phage of the type carried as prophage (BERTANI 1958), and stabilized CO₂-sensitive, rho, and ultra-rho strains are partially immune to infectious σ introduced by injection (L'HERITIER 1962). This immunity is also considered responsible for the generally low titer of infectious σ in stabilized sensitive strains compared to those which are nonstabilized.

The possibility should not be overlooked that *Dly* may be essentially analogous to "genetic virus" except that the former represents a chromosomally integrated form. In this hypothesis delayed recovery could be thought of as a special manifestation of the chromosomal agent, with or without the collaboration of the infectious agent, the controlled production of which is dependent on the gene rather than being maternally inherited. If eventually shown to be otherwise analogous to "genetic virus," chromosomally integrated *Dly* of strain TDR would represent an even closer relationship to the temperate phages than is found for stabilized sensitive, rho, and ultra-rho strains. Genetic elements such as temperate phages which can be present or absent and, if present, can be attached or free from the cell genome have been called episomes by JACOB and WOLLMAN (1958, 1961).

Preliminary studies indicating the existence of a partial immunity of the TDR strain to introduced infectious σ have been mentioned. A more convincing evidence relating *Dly* to "genetic virus" would be the conclusive demonstration that *Dly* elaborates the infectious agent in strain TDR. Good evidence that this is the case would be supplied if the agent could be demonstrated in the extract of heterozygous delayed-recovery progeny obtained from backcrosses of the type *resistant female* $\times$ *F₁ male* (resistant female $\times$ TDR male). Since σ is not inherited from males through two successive generations in *D. melanogaster* (L'HERITIER 1948), the presence of the σ -like agent in such progeny would show that it is not transmitted like σ in delayed-recovery strains, at least not exclusively. A few preliminary injections intended to show the presence of the infectious agent in these backcross progeny have not led to positive results, but the work is being continued. Since, as in rho flies, TDR individuals homozygous for *Dly* seem to only seldom produce detectable infectious particles, it would be quite possible that their production by heterozygous individuals, if it does take place, would be even more rare.

The hypothesis under consideration explains the origin and perpetuation of the infectious CO₂-sensitivity producing agent in the TDR strain. If the agent is involved in the delayed-recovery phenotype, the hypothesis would also account for the apparently nonmaternal inheritance of this phenotype. The hypothesis does not, however, at present offer an explanation as to how the infectious agent might be involved in the production of delayed recovery.

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SUMMARY

Delayed recovery to CO₂ represents a phenotype of the Texas Delayed Recovery strain (TDR), in which adults usually, though not always, recover from a standard 15 minute exposure to CO₂ at 14°C; but recovery is often not complete and generally requires from one to several hours rather than occurring within 15 minutes as is the case for resistant flies. The condition is primarily determined by a semidominant gene, designated *Dly*, which has been assigned to locus 52.7 of the third chromosome. Flies heterozygous for *Dly* all recover completely from CO₂ testing, with recovery generally requiring 15 minutes to 1 hour. A direct relationship exists between the duration of CO₂ exposure, the percentage of lethality and the time required for recovery of survivors. Recovery time is also longer and less certain as the testing temperature is increased. It is not possible to cure delayed recovery by heat treatment of adults, but a somatic cure of delayed-recovery symptoms can be accomplished by rearing the flies at 30°C. The pupal stage is most susceptible to this treatment. Adult delayed-recovery flies surviving a first exposure to CO₂ have been found to be much less severely affected by later exposures. Resistant flies have occasionally been made CO₂-sensitive by the injection of delayed-recovery fly extracts, and strains displaying maternally inherited CO₂-sensitivity were established from some of the induced sensitives produced in one injection experiment. This indicates the presence of an infectious σ -like agent in at least some delayed-recovery flies. Possible relationships between *Dly* and the cytoplasmic agent of strain TDR are discussed, including the possibility that *Dly* may be an episome.

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