THE α-GLYCEROPHOSPHATE CYCLE

IN *DROSOPHILA MELANOGASTER*

IV. Metabolic, Ultrastructural, and Adaptive

Consequences of *αGpdh-1* "Null" Mutations

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ABSTRACT

"Null" mutations previously isolated at the *αGpdh-1* locus of *Drosophila melanogaster*, because of disruption of the energy-producing α-glycerophosphate cycle, severely restrict the flight ability and relative viability of affected individuals. Two "null" alleles, $\alpha Gpdh-1^{BO-1-4}$, and $\alpha Gpdh-1^{BO-1-5}$, when made hemizygous with a deficiency of the *αGpdh-1* locus, *Df(2L)GdhA*, were rendered homozygous by recombination with and selective elimination of the *Df(2L)GdhA* chromosome. After over 25 generations, a homozygous $\alpha Gpdh-1^{BO-1-4}$ stock regained the ability to fly despite the continued absence of measurable αGPDH activity. *Inter se* heterozygotes of three noncomplementing *αGpdh-1* "null" alleles and the "adapted" $\alpha Gpdh-1^{BO-1-4}$ homozygotes were examined for metabolic enzymatic activities related to the energy-producing and pyridine nucleotide-regulating functions of the α-glycerophosphate cycle in *Drosophila*. The enzyme functions tested included glyceraldehyde-3-phosphate dehydrogenase, cytoplasmic and soluble malate dehydrogenase, lactate dehydrogenase, mitochondrial NADH oxidation, oxidative phosphorylation, and respiratory control with the substrates α-glycerophosphate, succinate, and pyruvate. These activities in any of the mutant genotypes in early adult life were indistinguishable from those in the wild type. There was, however, a premature deterioration and atrophy of the ultrastructural integrity of flight muscle sarcosomes observed by electron microscopy in the "null" mutants. These observations were correlated with a decrease in state 3 mitochondrial oxidation with α-glycerophosphate, succinate, and pyruvate, as well as with loss of respiratory control in adults as early as 2 wk after eclosion. Such observations, which normally are seen in aged dipterans, were accompanied by premature mortality of the mutant heterozygotes. The adapted $\alpha Gpdh-1^{BO-1-4}$ was identical with wild type in each of the aging characters with the single exception of lowered rates of mitochondrial oxidative phosphorylation.

The evolutionary requirement for both genic variability and recombinational plasticity provides a selective pressure on biological species in favor of mechanisms which preserve the temporary accumulation of mutational and recombinational variability. This selective pressure has produced and preserved certain biological "buffer systems" which protect spontaneous mutations and randomly produced gene combinations from the immediate rigors of natural selection (1, 2). These buffers include diploidy itself, functional redundancy (e.g., two biochemical routes to the same product), and retention of vestigal functions during ontogeny. A remarkable demonstration of such buffering is emphasized by the observation that of the 14 genes in *Drosophila melanogaster* at which "null" or "silent" alleles eliminate their respective gene products, only one, *bobbed*, has lethal alleles (3). "Null" alleles at each of the other gene-enzyme loci are all homozygous viable, despite the absence of presumably important functions. A particularly important function which exhibits such protection is the α-glycerophosphate (αGP) cycle, a major circuit in the energy-producing machinery of the insect flight muscle (4–8).

The αGP cycle consists of two biochemically and genetically distinct α-glycerophosphate dehydrogenases: a cytoplasmic NAD like α-glycerophosphate dehydrogenase (αGPDH; L-glycerol 3-phosphate:NAD oxidoreductase, EC 1.1.1.8), and a particulate mitochondrial inner membrane flavoprotein, α-glycerophosphate oxidase (α-GPO; L-glycerol 3-phosphate:cytochrome *c* oxidoreductase, EC 1.1.99.5). The αGP cycle provides an efficient means to effect three important functions: (*a*) maintenance of NAD-NADH equilibrium in the cytoplasm, (*b*) ATP production for muscular contraction, and (*c*) provision of αGP for phospholipid anabolism (4–6).

The two enzymes in *D. melanogaster* are products of different genes. The structural gene for αGPDH, designated *αGpdh-1*, is located on 2L at 2-20.5 (7, 9); αGPO is not affected by mutations at this locus but is sensitive to aneuploidy in a gene dosage response manner at a region (50C–52E) on 2R (10). This region probably contains the structural gene, although direct evidence is lacking. Despite the genetic distinctions, certain biochemical and developmental correlations between pairs of isozymes of the two enzymes suggest metabolic cooperation in the operation of the αGP cycle (6).

Four "null" or silent mutant alleles at the *αGpdh-1* locus were previously isolated after mutagenesis with ethyl methane sulfonate (EMS) (7).

Flies heterozygous for any of the noncomplementing alleles or hemizygous with a deficiency for the locus expectedly could not sustain flight. Two of these mutant stocks were carried as homozygotes for 6 mo before a surprising event occurred. *αGpdh-1^{BO}* "null" homozygotes were observed in the cultures of flies which flew for extended periods of time like their wild ancestors. Biochemical measurements confirmed that they still lacked any detectable αGPDH activity. Hence, a selective pressure had produced parallel adaptations in favor of flying ability by a mechanism independent of αGPDH activity.

Finally, it is well known that dipteran flies lose the capacity to fly with age (11). This deficiency is accompanied by an ultrastructural deformation of the large flight muscle mitochondria or sarcosomes (12, 13) in addition to age-dependent decreases in state 3 rates of oxidative phosphorylation and respiratory control ratios (RCR) (14). Hence, it was of some interest to examine the *αGpdh-1* mutants for a number of parameters other than αGPDH in an adult life profile. Because of the importance of the system as a principal sources of energy, and because of the nature of muscle metabolism and atrophy during aging, further investigation of the ultrastructural and biochemical consequences of *αGpdh-1* mutations seemed warranted. The observation of genetic adaptation of the mutants by alternative mechanisms for performing the three functions of the αGP cycle suggested the search for possible compensatory mechanisms in the repertoire of metabolic activity.

We report here a series of experiments designed to characterize more fully the phenotypic and/or metabolic effects of *αGpdh-1* mutations and to search for the metabolic basis of adaptation of the *α-Gpdh-1* "null" mutants.

MATERIALS AND METHODS

Stocks and Culture

The wild stock was homozygous for *αGpdh-1^B* and for *al*. The visible mutants and marker chromosomes are designated according to the conventions of Lindsley and Grell (15). All *αGpdh-1^{BO}* mutants were derived from the wild stock after chemical mutagenesis as described previously (7). *Df(2L)GdhA* is a deficiency of the *αGpdh-1* locus which includes *clot* (2-16.5) (9). The stock carrying the deficiency was generously provided by Dr. E. H. Grell.

Because of the potency of the EMS mutagenesis treatment, a number of recessive lethal mutations oc-

curred on each $\alpha Gpdh\text{-}1^{BO}$ chromosome at loci outside the limits of $Df(2L)GdhA$. This made the construction of homozygous "null" strains difficult (see reference 16); so, each null-containing chromosome was balanced over SM1, which contained Cy and al. De novo heterozygotes were constructed between two "null" alleles that have been maintained previously in a balanced stock with SM1. These have not had the opportunity to adapt in response to selective pressure.

Heterozygotes between noncomplementing mutants

were examined instead of homozygotes, because the EMS mutagenesis procedure induced lethal mutations on the null-containing chromosones at loci other than the $\alpha Gpdh\text{-}1$ locus, which made recovery of homozygous individuals impossible. Hemizygotes of different mutants with $Df(2L)GdhA$ were also unsatisfactory because the large deficiency in combination with the EMS-treated chromosomes produced lowered viability (S. J. O'Brien, unpublished observations).

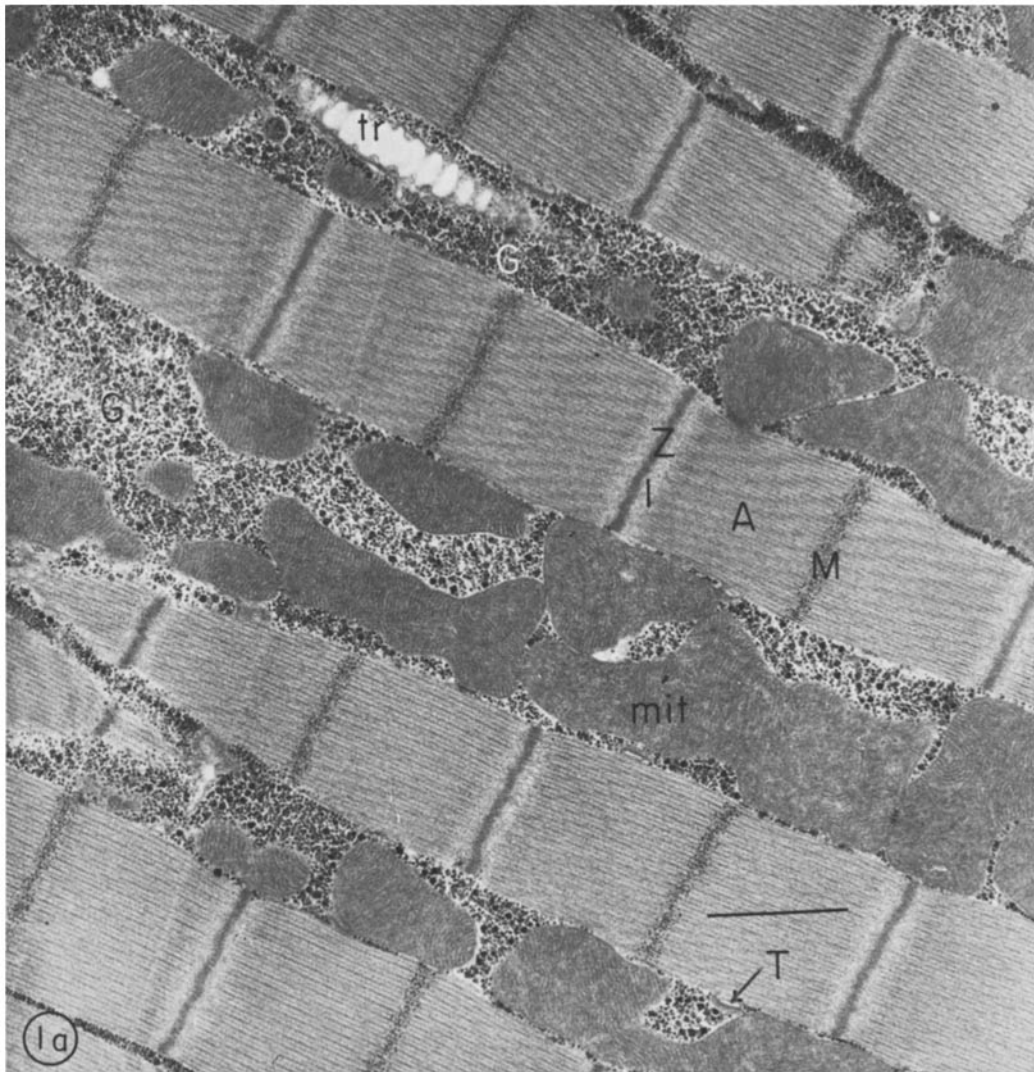The adapted or suppressed $\alpha Gpdh\text{-}1^{BO}$ strains were

FIGURE 1   Electron micrographs of sections of dorsal longitudinal flight muscle from 9-day old $+/+$ D. melanogaster. The myofibrils exhibit indicated muscle bands, surrounded by mitochondria (mit), glycogen granules (G), the transverse tubular (T) system, and tracheoles (tr). Fig. 1 a, Longitudinal section. Bar indicates 1 μm. × 19,500. Fig. 1 b, Cross section. Bar indicates 1 μm. × 9,970. In cross section the average of 21 measurements of the maximum mitochondrial length was 1.449 μm. The reader is referred to references 12, 13, and 23 for more detailed description of flight muscle ultrastructure.
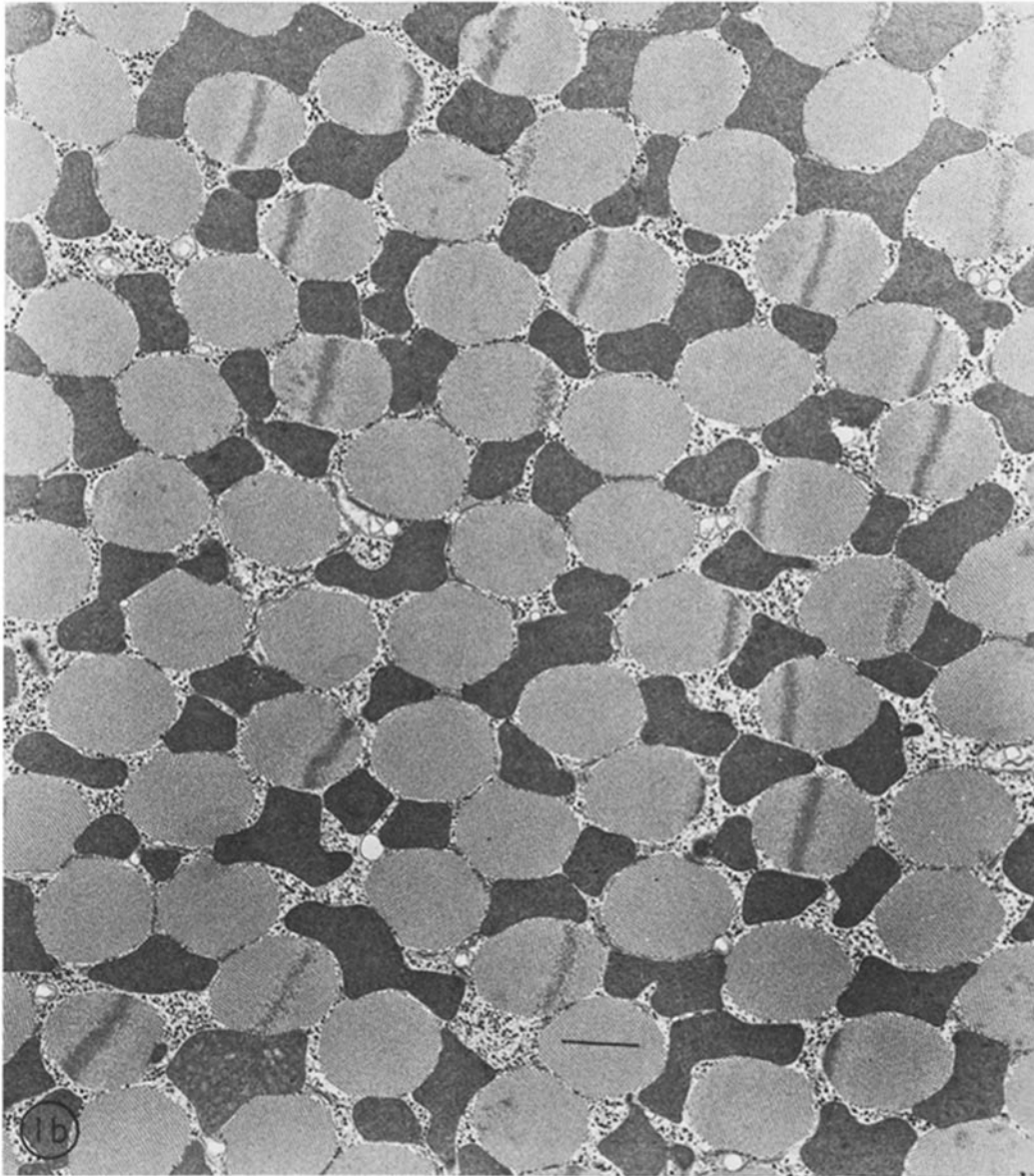
derived in the following way. Two of the null-containing chromosomes, $\alpha Gpdh\text{-}1^{BO\text{-}1\text{-}4}$, or *BO-1-4*, and $\alpha Gpdh\text{-}1^{BO\text{-}1\text{-}5}$, or *BO-1-5*, were made hemizygous with *Df(2L)GdhA* and allowed to breed in separate cultures for 15 generations. After 15 generations the stocks were examined for αGPDH, flight ability, and the presence of *Df(2L)GdhA*. The presence of *Df(2L)GdhA* was detected genetically by crossing 200 single members of the adapted strain with *clot* virgin females. The presence of *clot* offspring indicated the transmission of *Df(2L)GdhA*; the presence of *clot*$^+$ indicated transmission of the *clot*$^+$ allele and thus the null allele from the original $\alpha Gpdh$-

1^{BO} chromosome. Flying ability was determined as described previously (7).

Flies were grown at 25°C on a high sucrose-yeast extract medium in the absence of chloramphenicol (17). The mortality estimates were obtained by transferring emerging offspring of each genotype to fresh bottles (approximately 30 flies/bottle) within 24 h of emergence with weekly medium transfer.

Mitochondrial Oxidative Phosphorylation

Mitochondria which exhibited tight coupling of electron transport after pyruvate oxidation were isolated as

follows. Staged adults were cold-anesthetized and gently ground in 10 vol (assume 1 g flies equals 1 ml) of isolation medium (0.01 M potassium phosphate, 0.001 M Na₂-EDTA, 0.38 M sucrose, 0.5% bovine serum albumin [BSA] [18]). This brei was diluted 10 times with isolation medium (IM) and filtered through three layers of cold IM-soaked surgical gauze to remove body integuments. The filtrate was centrifuged at 12,000 g. The supernate was decanted, and the pellet containing the mitochondria was washed once more and resuspended in 7.5 times the original volume of flies.

Respiratory rates were measured polarographically at 30°C with a Clark oxygen electrode. Mitochondrial protein was determined by the Lowry method with BSA as a standard. Between 200 and 800 µg of mitochondrial protein were incubated in a final volume of 2 ml in 0.133 M KCl, 0.01 M HEPES, 0.01 M potassium phosphate (pH 7.1), 0.1% BSA, 0.5 mM MgCl₂, and 0.5 mM ADP. The substrates were present at concentrations at least three times their apparent K_m values; 1 mM for pyruvate, 5 mM for proline, 5 mM for succinate, 50 mM for αGP. After reaction mixes were brought to temperature equilibrium, mitochondria, substrate, and ADP were added. State 3 rates were observed, and when ADP became limiting, state 4 rates were recorded. Then 1 µmol of ADP was again added initiating state 3 rates. The ratio of state 3 rates (excess ADP) to state 4 rate (ADP limiting) is the RCR. The ADP/O ratio is the moles of ADP added divided by microgram atoms of oxygen consumed during state 3 (19). This value is an indication of P/O ratio and therefore of phosphorylation sites involved with different substrates.

Enzyme Assays

αGPDH: The soluble αGPDH and the mitochondrial αGPO were assayed spectrophotometrically at 30°C with a Gilford 2000 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). αGPDH was measured by NADH production and αGPO by tetrazolium reduction in solution as described (6).

MALATE DEHYDROGENASE (MDH): Both mitochondrial and soluble MDH were assayed spectrophotometrically after NADH oxidation in response to oxaloacetate reduction as described (20).

NADH OXIDASE: Mitochondrial oxidation of NADH was measured with intact mitochondria prepared as above. The reaction proceeded in the polarographic mixture with the addition of exogenous NADH (30 mM) to start the reaction. MDH mediated uptake and oxidation of NADH was monitored by the addition of 0.8 mM oxaloacetate to this mixture. The oxidation of NADH was followed spectrophotometrically at 340 nm.

LACTATE DEHYDROGENASE (LDH): The assay for LDH was developed with larvae, because *Drosophila* adults have very little, if any, LDH (21). 100 mg of larvae were homogenized in a ground-glass tissue grinder in 0.5 ml 0.05 Na pyrophosphate, pH 8.3. This material was

centrifuged in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and the crude supernate was used as a source of LDH. 100 µg soluble protein were added to a cuvette containing 0.1 M potassium phosphate (pH 7.5), 0.83 mM NAD, and 0.83 mM lithium lactate. NADH production was followed spectrophotometrically at 340 nm.

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE: Flies were homogenized in 10 vol of 0.1 M Tris-HCl (pH 8.5) containing Na₂EDTA (0.001 M). This material was centrifuged in a Beckman microfuge and the supernate was recovered. 25–100 µg were added to a cuvette containing 0.1 M Tris-HCl (pH 8.1), 0.001 M EDTA, 0.67 mM NAD, 0.0087 M Na arsenate, 90 mM β-mercaptoethanol, 4.0 mg/ml gelatin, 1.5 mg/ml phenazine methosulfate, and 4.0 mg/ml p-iodonitro tetrazolium violet. The reaction was initiated by the addition of glyceraldehyde-3-phosphate (2.64 µmol) which had been converted from the diacetal barium salt to a sodium salt according to the instructions described by Sigma Chemical Company Technical Bulletin no. 10, 1961. Tetrazolium reduction was followed spectrophotometrically at 490 nm (6).

Electron Microscopy

Flies were cold-anesthetized in Petri dishes and placed in a drop of fixative consisting of 2.5% glutaraldehyde in 0.05 M cacodylate buffer-0.18 M sucrose, pH 7.4 (22). The heads and abdomens were carefully removed, and the thoraces were transferred to bottles containing fixative and shaken for 20 min. At this time each thorax was bisected with a razor blade and returned to the fixative for an additional 2 h and 40 min at 0°-4°C. The material was then fixed, sectioned, and stained as described previously for blowfly flight muscle (12).

Cross sections of at least four staged adults' muscles of various genotypes were examined and photographed at relatively low power ($\times$ 6,900) where 1–200 sarcosomes could be observed in the field. The presence of at least five damaged mitochondria per field was recorded as the beginning of mitochondrial atrophy.

RESULTS

Adaptation of $\alpha Gpdh-1^{BO}$ Null Alleles

The induction and isolation of four $\alpha Gpdh-1^{BO}$ "null" allele-containing chromosomes (*BO-0, BO-5-4, BO-1-4,* and *BO-1-5*) have been described (7). *BO-1-5* is leaky with respect to αGPDH activity and exhibits interallelic complementation with *BO-0* and *BO-1-4* (7). Because the "null"-containing chromosomes also contained EMS-induced background lethal mutations at other loci which prevented the immediate homozygosity of mutant chromosomes, each chromosome was balanced over SM1. Flies deficient in αGPDH were

produced as hemizygotes over *Df(2L)GdhA* or as *inter se* "heterozygotes" of different null alleles. Flies thus constructed had low viability and could not sustain flight for over a few seconds (7, 8). Lethal genes on each of these chromosomes were complementing in all but one case, *BO-1-5/ BO-1-4* (7), permitting stock construction. In order to establish stocks which were homozygous for the "null" alleles, virgin *Df(2L)GdhA/BO-1-4* and *Df(2L)GdhA/BO1-5* hemizygotes were constructed and sibmated. The expectation was the segregational loss of the deficiency-carrying chromosome because of aneuploid viability decrease followed by selection of "null"-containing chromosomes. The remaining chromosomes subsequently either segregated their lethal genes after recombination with the *Df(2L)GdhA* or produced complementing lethal-containing chromosomes with the "null" region present and homozygous.

In the initial generations of the experiment, the viability of these crosses was suboptimum, and the offspring were incapable of sustained flight. However, after 12 mo of serial transfer both stocks were characterized by flies whose flying ability was indistinguishable from that of wild type. Single flies were examined genetically for the presence of *Df(2L)Gdh* by crosses to *clot* females. All F_1 were *clot*⁺ in both lines, indicating the loss of the deficiency. The flies were then examined in multiple fly homogenates for αGPDH activity. *BO-1-5* homozygotes had 15–25% wild type activity, which could account for the flight recovery (7). The *BO-1-4* individuals, however, had negligible amounts ($\leq$0.1% wild type levels of αGPDH). Hence, an adaptation or a suppressor had been selected in the 6 mo of breeding which compensated for the flight deficiency despite the genetic diminution of αGPDH. The adapted *BO-1-4/ BO-1-4* stock was subsequently designated as *BO-1-4,S(G)* where the *S(G)* designates dominant suppression of αGpdh-1. The suppression was dominant and nearly monomorphic in the stock as demonstrated by crossing *BO-1-4,S(G)* × *SM1/ Df(2L)GdhA*. Of the *Cy*⁺ offspring, 85% were capable of flight (n = 85) compared to 91% (n = 60) for wild type αGpdh-1ᴮᴮ homozygotes of the same age.

Ultrastructural Studies of Flight Muscle of αGpdh-1ᴮᴼ Mutants

An ultrastructural analysis of indirect flight muscle in staged adult *Drosophila* mutants was undertaken for two reasons. First, the αGP cycle

exerts an important effect on the contraction of normal flight muscle. Mutants therefore might be expected to exhibit pleiotropic effects on the flight muscle and sarcosome integrity not observed previously. Second, since senescent flies normally lose flying ability in concert with flight muscle deterioration and atrophy, it was of interest to examine flies genetically deficient in flight for an apparently different reason.

Flight muscle sections of wild type *D. melanogaster*, de novo produced heterozygotes between the three noncomplementing "null" alleles, and *BO-1-4,S(G)* were examined at 3, 9, 14, 17, 25, and 31 days after eclosion. Young wild type flight muscles (Fig. 1) exhibited characteristic myofibrils, A, I, M, and Z bands, large sarcosomes with elaborate inner membrane, tracheoles, and glycogen deposits as previously described (13, 23). In the first week after eclosion all mutant flies were indistinguishable from wild type. Wild type preparations maintained this appearance for longer than 31 days before any age-dependent mitochondrial deterioration was observed. *De novo* mutant combinations, however, exhibited general mitochondrial disintegration at much earlier stages (Table I and Figs. 2–4) and continued to deteriorate at later ages. This disintegration was very similar to the age-dependent atrophy seen in older flies (12, 13) and was characterized by membranous whorls, glycogen rosettes, and reorganization of the inner membrane.

Although the three *de novo* mutant heterozygotes were characterized by premature mitochondrial degeneration, the *BO-1-4,S(G)* strain maintained normal ultrastructure for over 30 days (Fig. 5). This mutant line, which has circumvented the flight restriction, has also overcome the ultrastructural consequence of αGPDH diminution.

TABLE I

Post Eclosion Age when Degenerative Changes were First Observed in Flight Muscle of αGpdh-1ᴮᴼ *Mutants*

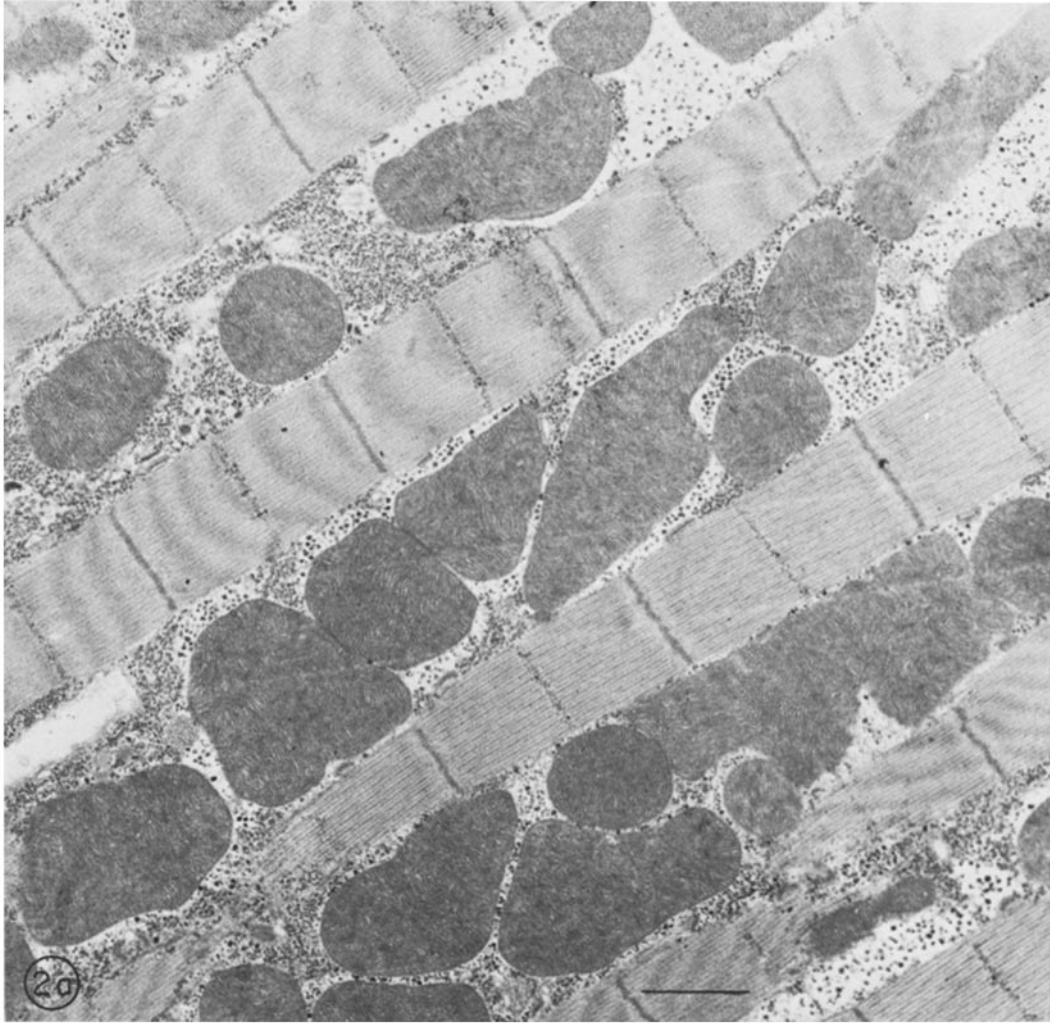|	Genotype	Age
		Days
	+/+	>31
	BO-1-4,S(G)	>31
	BO-1-4	
	BO-0/BO5-4	14–17
De novo Hets*	BO-5-4/BO-1-4	9–14
	BO-0/BO-1-4	4–9

* *De novo* Hets, see Materials and Methods.

FIGURE 2   Electron micrographs of *BO-0/BO-1-4* at different ages post eclosion. Fig. 2 *a*, 4-days old. Bar indicates 1 μm. × 13,900. Fig. 2 *b*, 9-days old, low power micrograph. Bar indicates 5 μm. × 5,460. Fig. 2 *c*, high power micrograph of same preparation as in Fig. 2 *b*. Bar indicates 1 μm. × 32,000. Fig. 2 *d*, beginning of mitochondrial atrophy of same preparation as Fig. 2 *b*. Note swellings of mitochondrial inner membrane. Bar indicates 0.5 μm. × 50,700.

Energetics of αGpdh-1^{BO}

The huge sarcosomes of insect flight muscle are composed largely of mitochondrial inner membrane as compared to outer membrane and matrix (Fig. 1). This differentiation is reflected by the presence of those enzymes concerned with oxidative phosphorylation on the inner membrane (24) and the tremendous energy expense associated with wing movements during flight. To determine if energy generation was also impeded in the αGpdh-1^{BO} mutants, as seen in senescent flies (14), oxidative phosphorylation was measured in vitro for three substrates: pyruvate + proline (25, 26), succinate, and αGP. The measurements were made immediately after eclosion and 3 wk later when each of the mutants exhibited sarcosomal atrophy.

Table II presents state 3 (excess ADP) and state 4 (limiting ADP) rates of O_2 consumption, RCR, and ADP/O ratios of wild and mutant strains at
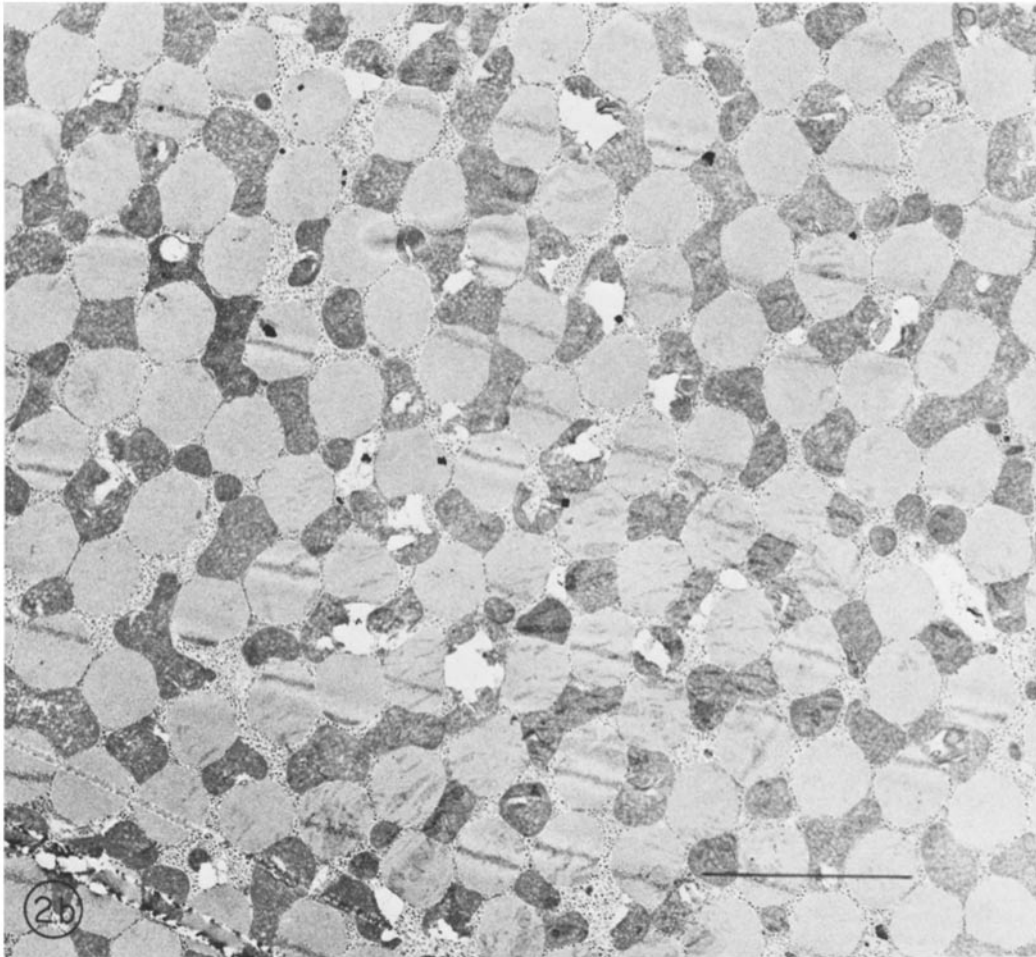
2-5-days old and 21 days after eclosion in response to pyruvate + proline. This reaction was tightly coupled (state 4 rates were barely measurable), and exhibited a P/O ratio near 3, indicating that pyruvate enters the respiratory chain at site 1. Both the mutant and adapted $\alpha Gpdh-1^{BO},S(G)$ fly mitochondria exhibited measurements not significantly different from that of wild type in flies less than 5-days old. However, in mitochondria from individuals staged to 21 days after eclosion severely diminished state 3 rates were observed in both *de novo* $\alpha Gpdh-1^{BO}$ heterozygotes and in the *BO-1-4,S(G)* stock. These rates were between 50 and 60% of wild type rates from flies of the same age. Mutants' ADP/O ratios were similar to wild type, and the higher RCR in older *de novo* heterozygotes suggest even lower state 4 rates.

The oxidations of αGP and succinate by the same wild type mitochondrial preparations were loosely coupled or not at all to ADP phosphorylation. This observation could be explained in at least two ways. The first possibility is that substrates like pyruvate, which enter the respiratory chain at site 1, are more tightly coupled than those which enter at site 2 as do succinate and αGP. An alternate possibility is that pyruvate is only oxidized by pyruvate dehydrogenase complexes which have tightly coupled respiratory chains available, while succinate and αGP oxidation are not so restricted. Hence, succinate and αGP can be oxidized by damaged and uncoupled mitochondria while pyruvate cannot, possibly as a consequence of their entry position on the cytochrome chain. Because of the lack of ADP dependence for suc-

cinate and αGP, meaningful respiratory control rates or ADP/O ratios could not be calculated.

Table III presents the "state 3" [1] rates of mitochondria from the five genotypes at different ages in response to αGP and succinate. The older mutants (21 days) had significantly lower rates of both αGP and succinate compared to wild type controls of the same age. This deficiency was also seen in the *BO-1-4,S(G)* individuals.

Mortality of αGpdh-1[BO] Mutants

Since the mitochondrial degeneration and dysfunction are normally observed in senescent flies,

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[1] We call them state 3 because initial ADP stimulation is required for initiation of O_2 consumption. However, there is little or no sharp cutoff of respiratory rate at a later time.

mortality estimates were prepared for each genotype (Fig. 6). Wild type flies had a median survival time of 40 days under the conditions of the experiment. *De novo* mutants had median survival of 14, 20, and 30 days after eclosion, while *BO-1-4,S(G)* had a longevity indistinguishable from that of wild type.

Search for Molecular Compensation for αGpdh-1[BO] Mutations

Because of the important functions of the αGP cycle in the flight muscle, it would be of considerable interest to detect the compensatory mechanisms which permit survival of the *de novo* heterozygotes, as well as the mechanism of suppression of the αGpdh-1[BO] phenotypes observed in the *BO-1-4,S(G)* mutation. A number of enzyme
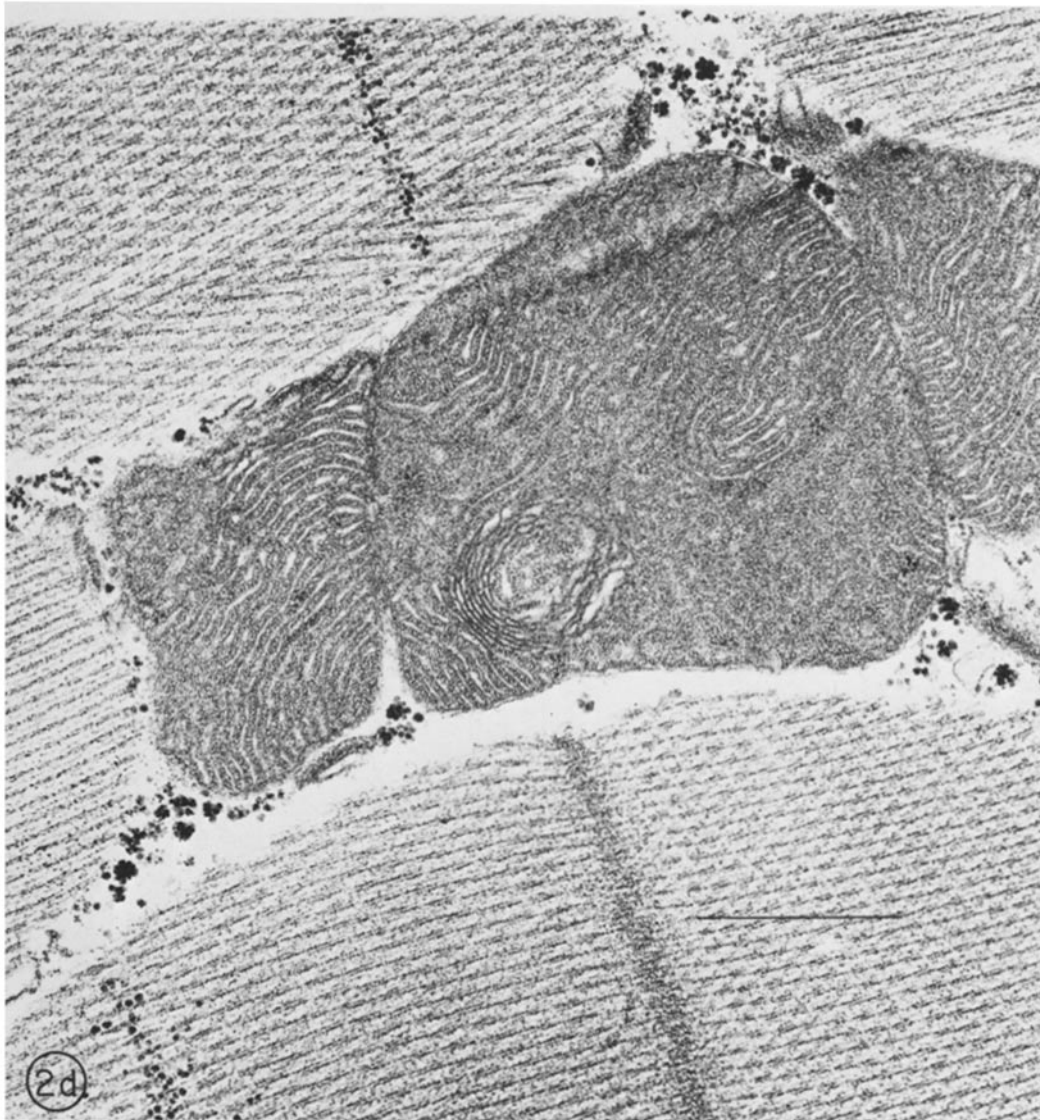
systems which relate to, or could conceivably compensate for, the three functions of the αGP cycle were examined in the five genotypes described above.

The mitochondrial αGPO was examined because it is the normal half of the cycle in mutant flies. Glyceraldehyde-3-phosphate dehydrogenase was examined because this is the enzyme in glycolysis which will be affected if NADH becomes limiting due to αGPDH dysfunction. A possible change in the permeability of mitochondria to pyridine nucleotides (27) was measured by following exogenous NADH oxidation by intact mitochondria. LDH is the enzyme used in mammalian systems to generate cytoplasmic NADH. In *Drosophila*, LDH is present in large amounts in larvae but is virtually absent in adults (21, 28). This enzyme, if reexpressed in adults, could compensate for the $\alpha GPdh$-1^{BO} mutations. There is a MDH cycle postulated in insects which could also compensate for the mutations (29). Thus both cytoplasmic MDH and mitochondrial MDH mediated NADH oxidation were examined. A possible source of ATP would be the citric acid cycle. Hence, the data in Tables II and III measuring pyruvate, succinate, and αGP were reexamined.
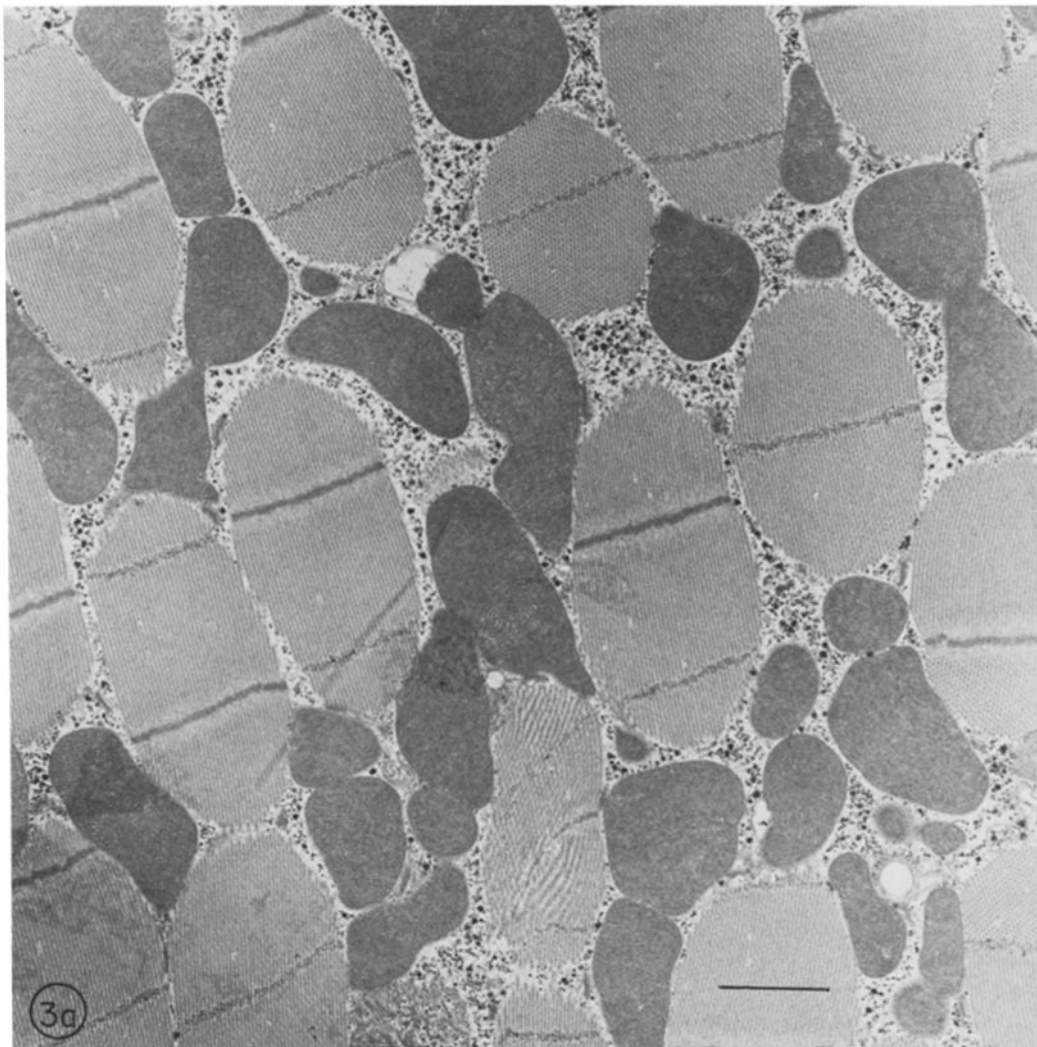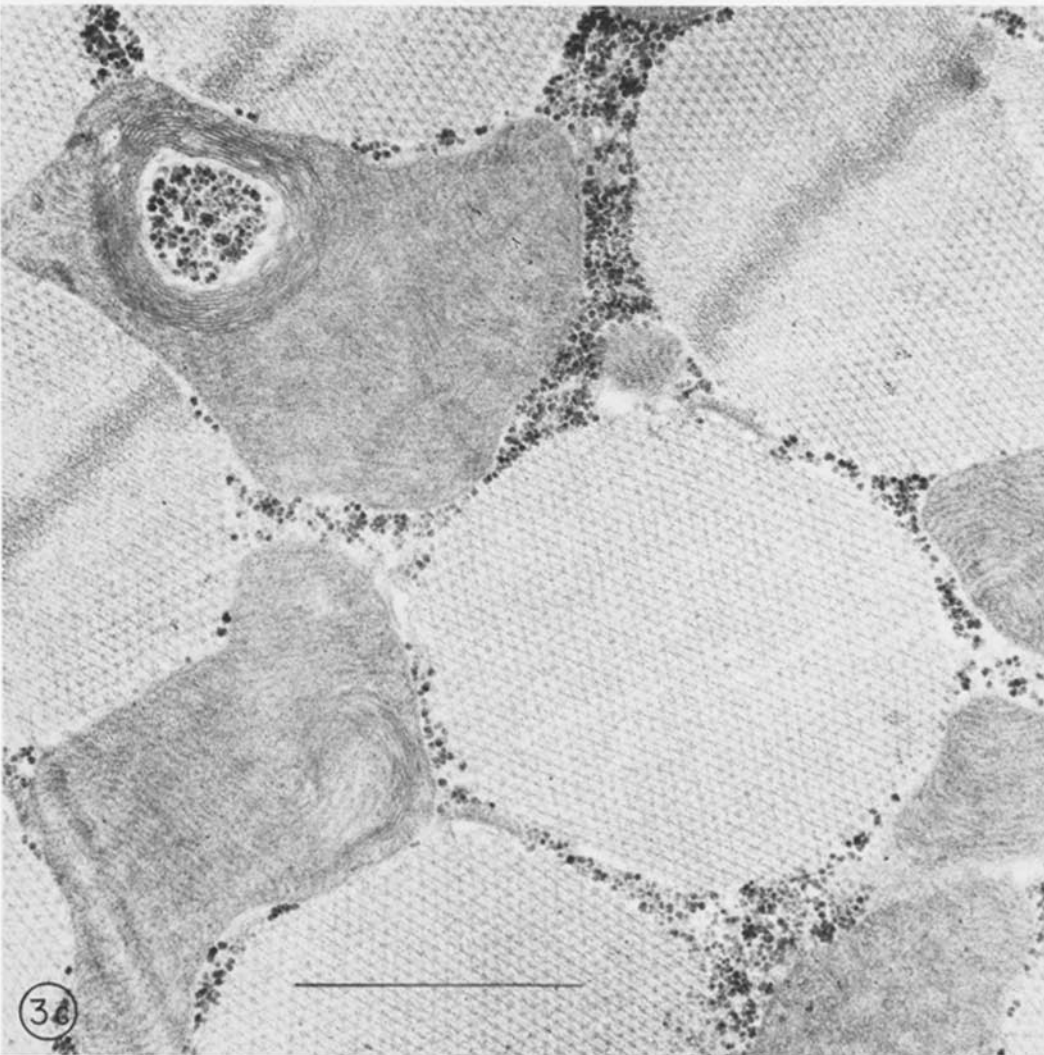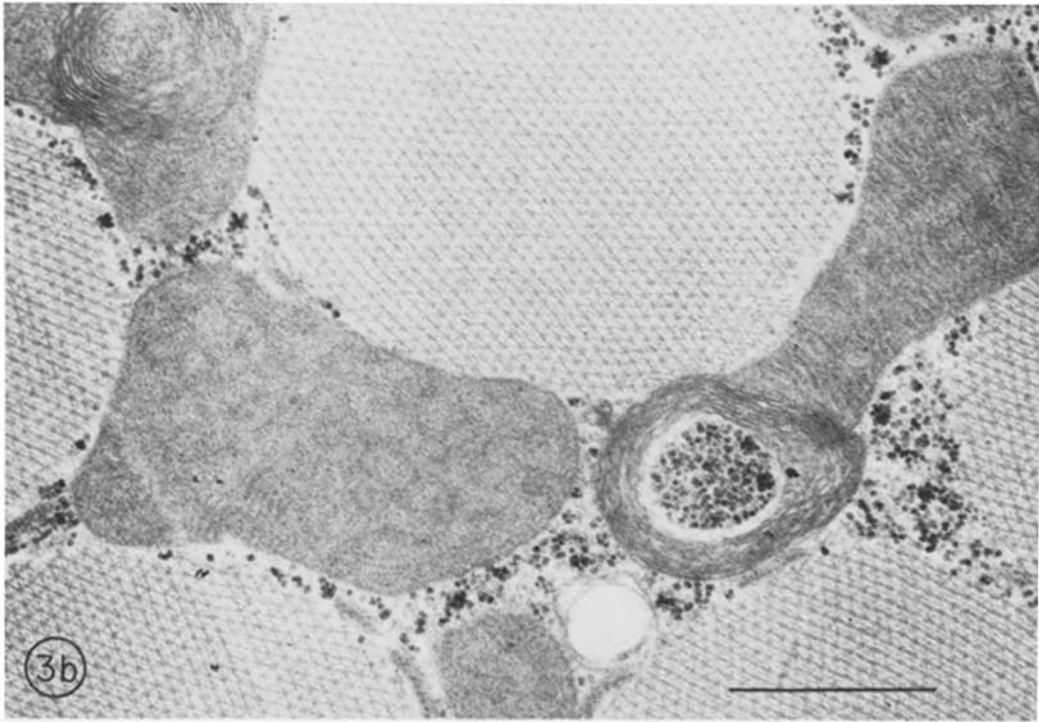
FIGURE 3 *BO-1-4/BO-5-4* flight muscle sections at three ages post eclosion. Fig. 3 *a*, 4-days old. Bar indicates 1 μm. $\times$ 14,200. Fig. 3 *b*, high power micrographs at 21 days. Bar indicates 0.5 μm. $\times$ 51,100. Fig. 3 *c*, 25 days. Bar indicates 1 μm. $\times$ 37,300. Note glycogen inclusions in mitochondria of Figs. 3 *b* and *c*.

A reexamination of Tables II and III did not reveal any highly significant increase in the mutants or in *BO-1-4,S(G)* over wild type. There was a reproducible increase in activity in each of the substrates in the *BO-0/BO-1-4 de novo* heterozygotes. This increase could be interpreted as a multigenic compensation, but it is equally likely to be a lowering of mitochondrial protein in the preparations from this stock. Furthermore, the dramatic adapted strain *BO-1-4,S(G)* did not exhibit elevated rates with any of the substrates. A compilation of the enzyme activities of each of the postulated compensatory mechanisms is presented in Table IV. None of the activities in the mutants was distinguishable from the corresponding wild type activity. Hence, within the experimental limits of our assay procedures, these enzymes did not appear to be capable of compensating for the rapid and efficient production of ATP and NAD attributed to the αGP cycle in dipterans (5, 29).
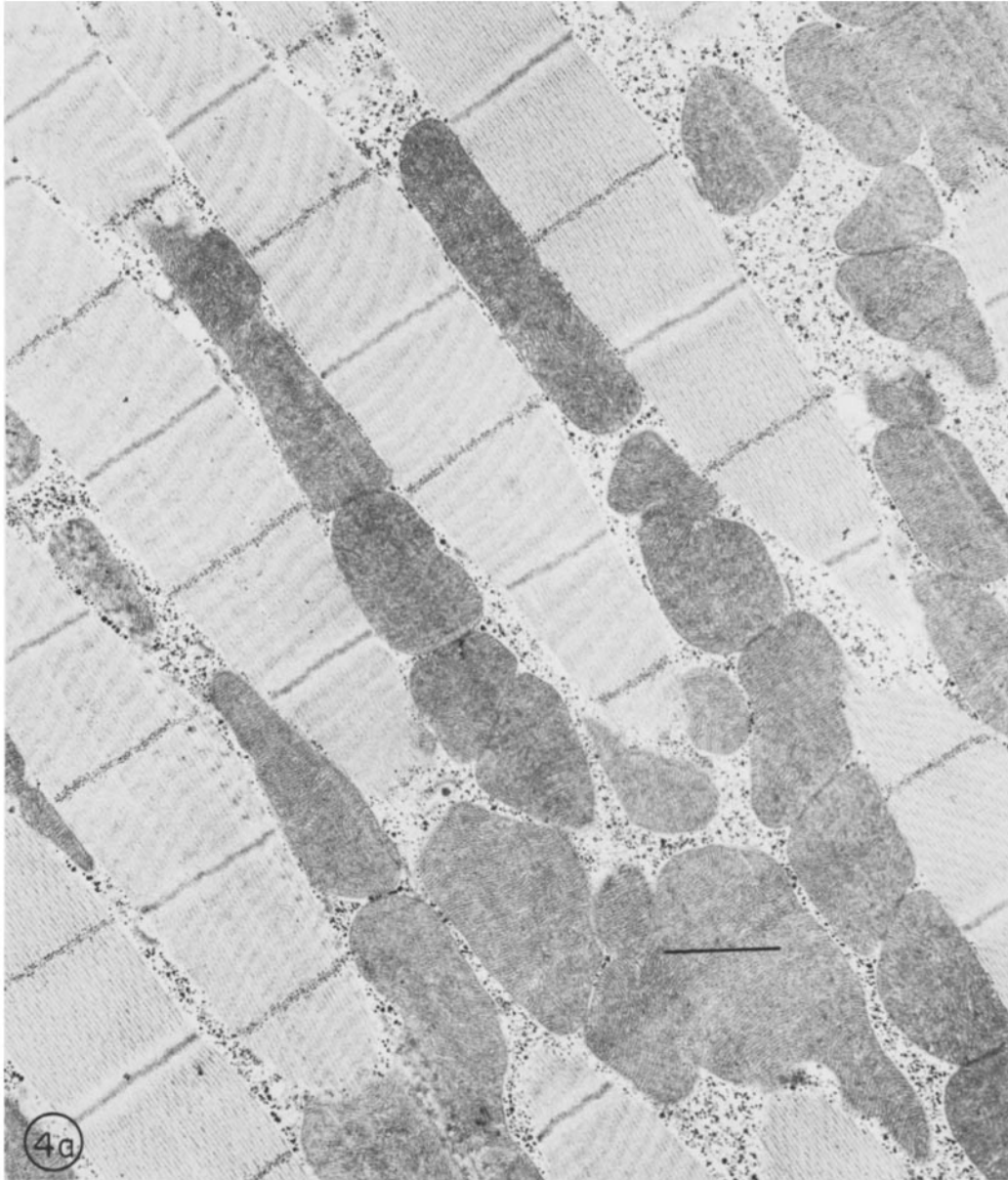
FIGURE 4 *BO-5-4/BO-0* flight muscle sections at two ages post eclosion. Fig. 4 *a*, 4 days. Bar indicates 1 μm. × 14,800. Fig. 4 *b*, 31 days. Bar indicates 1 μm. × 16,200.

DISCUSSION

The results presented here and earlier bear upon very different aspects of physiological genetics. We shall attempt to discuss below the technical, physiological, and evolutionary significance of these experiments in turn.

The Free Recombination Scheme

We have presented a simple and effective means of eliminating background lethals on mutagenized chromosomes by free recombination with a small deficiency. This procedure depends upon the availability of a deficiency which covers the region

which is to become homozygous. This condition is not limiting, however, in light of the recent availability of *Drosophila* translocations which permit construction of very small deficiencies and covering approximately 85% of the autosomes (10, 30) and one-third of the X chromosome (31). The scheme requires only one cross followed by mass culture over several generations. The alternative procedure of crossing mutants to marked stock and monitoring crossing over takes at least six generations just for a terminal chromosome region (16). The free recombination scheme does depend, however, upon the selective disadvantage of the deficiency relative to the mutagenized chromo-
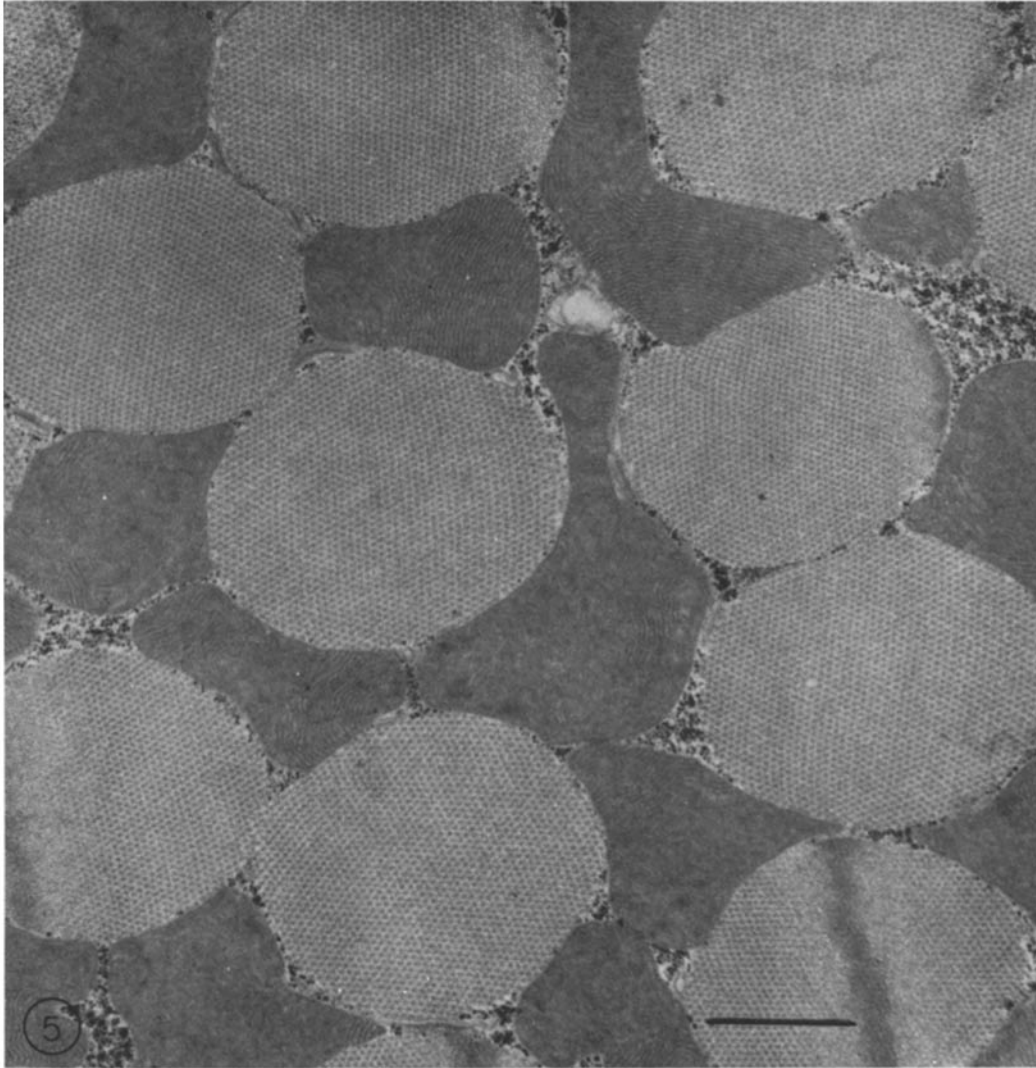
FIGURE 5 *BO-1-4/BO-1-4;* S(G) flight muscle sections at 25 days after eclosion. Bar indicates 1 μm. ×
19,400.

some. Furthermore, there is no guarantee that the lethals will be altogether eliminated, for they could merely be rearranged into complementing lethal chromosomes.

Pleiotrophy at the αGpdh-1 Locus

The physiological effects of *αGpdh-1*[BO] mutants are numerous but certainly related. Mutants have a lowered relative viability and an inability to sustain flight. They also are characterized by a syndrome of premature senescence, as can be seen in their mitochondrial deterioration, diminished rates of oxidative phosphorylation, and early mortality. This mitochondrial dysfunction occurs in flies deficient in a cytoplasmic enzyme, αGPDH, which is genetically coded by a gene distinct from the locus for the mitochondrial αGPO (7, 10). One possible explanation for such a situation would be a disuse hypothesis. αGPO, which is located on the mitochondrial inner membrane, is one of the most active of sarcosomal enzymes (29). Its substrate, αGP, is generated by the soluble αGPDH. The mutants presumably have limiting amounts of this metabolite and decreased functioning of a αGP

TABLE II

Respiratory Rates* of $\alpha Gpdh\text{-}1^{BO}$ Mitochondria Pyruvate Plus Proline

	Genotype	2-5 days				20-22 days		
		State 3	State 4	RCR	ADP/O	State 3	State 4	RCR
	+/+	1.18	0.11	10.7	2.45	1.29	0.27	4.78
		1.0	0.27	3.7	2.35	1.16	0.11	10.5
		0.88	—	—	—	0.94	—	—
	BO-1-4,S(G)	0.93	0.164	5.7	2.8	0.685	0.09	7.61
	BO-1-4	0.90	0.09	10.0	2.7	0.59	0.05	11.8
		0.66	—	—	—	0.48	—	—
	BO-0/BO-1-4	1.23	0.13	9.5	2.84	0.67	0.032	20.93
		1.1	0.128	8.6	2.70	0.63	0.005	126
		1.1	—	—	—	0.51	—	—
De novo Hets	BO-5-4/BO-1-4	1.08	0.155	7.0	2.57	0.75	0.032	23.4
		1.13	0.155	7.3	2.45	0.62	0.005	124
		0.87	—	—	—	0.52	—	—
	BO-0/BO-1-4	1.64	0.282	5.8	2.7	0.737	0.02	36.8
		1.48	0.201	7.4	2.45	0.623	0.008	78
		1.28	—	—	—	0.45	—	—

* Microgram atoms O_2 per minute per milligram mitochondrial protein. Values are actual measurements of the same preparation primed by the addition of ADP. Duplicate measurements of the same preparation were identical. Duplicate preparations gave results which were deviant in absolute values by relatively identical within the same experiment.

TABLE III

Respiratory Rates of $\alpha Gpdh\text{-}1^{BO}$ Mitochondria

	Genotype	αGP state 3 rate*		Succinate state 3 rate*	
		2-5 days	20-22 days	2-5 days	20-22 days
	+/+	1.74	1.81	0.198	0.245
	BO-1-4,S(G)	2.31	0.81	0.226	0.165
	BO-1-4				
	BO-0/BO-1-4	2.62	0.58	0.242	0.134
De novo Hets	BO-5-4/BO-1-4	2.15	1.02	0.199	0.178
	BO-0/BO-1-4	3.85	0.84	0.338	0.127

* Microgram atoms O_2 per minute per milligram mitochondrial protein. See text for explanation of state 3. Values are averages of four measurements from a single preparation, the same as is presented in Table II. Duplicate preparations gave results which were deviant in absolute values but relatively identical within a given experiment.

oxidation. If the rapid turnover of the flavoprotein and cytochomes in response to αGP is necessary for structural integrity of sarcosomes, then $\alpha Gpdh\text{-}1^{BO}$ mutations would result in age-dependent mitochondrial atrophy.

Adaptation

The survival of $\alpha Gpdh\text{-}1^{BO}$ mutants is a cogent demonstration of biological buffering which provides alternative routes to the same end. The adaptation of the BO-1-4,S(G) line to the ability to fly despite malfunction of the αGP shuttle is an even more striking example of the genetic plasticity of Drosophila. This adaptation, however, is not absolute. Although the BO-1-4,S(G) can fly by our test and has an extended life-span compared to unsuppressed mutants, its mitochondrial respiratory rates are diminished in the older flies compared to wild type. This diminished mitochondrial function is in spite of normal ultrastructure of

flight musculature 4 wk later. The interpretation of these results is difficult without further experimentation, but they could be the result of malfunctioning ubiquitous mitochondria compared to good sarcosomes, since both types are present in the mitochondiral preparations.

FIGURE 6   Survival curves for $\alpha Gpdh\text{-}1^{BO}$ mutants aged under conditions described in Materials and Methods. The genotype and number of flies scored in each experiment is indicated.

It is of some interest to point out that the opposite adaptation in favor of flight inability has been reported for insects which inhabit oceanic islands (1). The fliers in these populations are swept out to sea by the high winds, conferring on them a rather dramatic selective disadvantage. It is not difficult to surmise the selective advantage involved in the $S(G)$ adaptation since the net result is life extension and hence increased reproductive opportunity and fitness.

Whether the adaptation of the $BO\text{-}1\text{-}4,S(G)$ flies represents a single gene suppressor or selection for numerous compensating genes will not be clear until the results of the genetic analysis are complete (S. J. O'Brien, unpublished observations). If the suppression is unigenic, it should be easily mapped by conventional Drosophila procedures. A multigenic suppression, however, would be more difficult to analyze genetically. In fact, DDT resistance in Drosophila has been shown to be multigenic, and in each case a different physiological mode of adaptation was observed (32, 33). It is quite likely that a number of compensatory mechanisms may also be available for the functions of the αGP cycle.

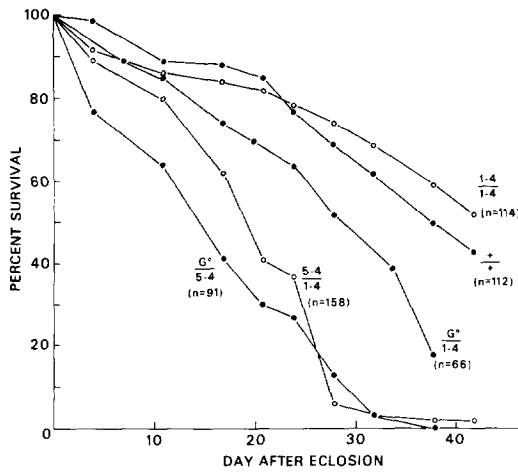The failure to detect an unequivocal compensation could be the result of one or a combination of

TABLE IV

Enzyme activities* of $\alpha Gpdh\text{-}1^{BO}$

		Soluble enzymes			
	Genotype	αGPDH	G3PD	LDH	cMDH
	+/+	442 ± 2.5	1,242 ± 66	6.7 ± 0.9 (larvae 82.6 ± 9.6)	4,355 ± 186
	$\dfrac{BO\text{-}1\text{-}4,S(G)}{BO\text{-}1\text{-}4}$	0.0 (<0.8)	1,515 ± 262	6.2 ± 0.5	4,715 ± 396
De novo Hets	BO-0/BO-1-4	11.9 ± 0.3	1,339 ± 449	7.8 ± 0.8	7,130 ± 256
	BO-0/BO-5-4	16.9 ± 0.6	1,555 ± 39	5.1 ± 1.3	4,530 ± 520
	BO-5-4/BO-1-4	14.0 ± 0.4	1,270 ± 220	5.0 ± 0.8	5,175 ± 1,160

		Mitochondrial enzymes		
	Genotype	αGPO	NADH oxidation	Malate dependent NADH oxidation
	+/+	6,300 ± 438	50.4 ± 20.7	26.0 ± 10.1
	$\dfrac{BO\text{-}1\text{-}4\text{-},S(G)}{BO\text{-}1\text{-}4}$	6,107 ± 561	25.3 ± 2.7	35.1 ± 10.6
De novo Hets	BO-0/BO-1-4	6,060 ± 671	18.5 ± 2.3	5.5 ± 3.0
	BO-0/BO-5-4	5,720 ± 275	38.7 ± 20	1.4 ± 2.0
	BO-5-4/BO-1-4	5,470 ± 356	26.4 ± 12.1	3.2 ± 3.0

* Nanomoles per minute per milligram protein (± SE).

several problems. We cannot exclude the back mutation of the αGpdh-1 locus which produces a physiologically active enzyme which is sensitive in the extraction procedures. The compensation could involve selection for several small changes in the measured systems which are not detectable within the experimental error. This possibility would be likely if differential gene expression for compensatory mechanisms only affected sarcosomes and not mitochondria in other tissues which are present and active in our mitochondrial preparations.

Possible compensatory mechanisms related to lipid metabolism have not been tested in this study. An alternative pathway, the pentose phosphate shunt, could bypass glycolysis entirely and obviate the need for NAD, while simultaneously generating ATP. There are also other shuttles reported in mammalian systems which could be involved in the transport of reducing equivalents into mitochondria, e.g., the fatty acid shuttle (34) and the purine nucleotide shuttle (35). An entirely new energy source, fatty acids, could be utilized in the flight muscle of these mutants. Fatty acids and not carbohydrates, serve as a primary ATP source in Lepidoptera, Orthoptera, and Homoptera (36, 37). If dipterans retain a vesigal circuit for fatty acid utilization from ancestral origins, the reactivation of such systems could solve the problem summarily. Further interpretation of the basis of compensation would be speculative at best and must await genetic and metabolite level studies in these mutants.

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