Archives of Insect Biochemistry and Physiology 1:243–250 (1984)

Ten Generations of *Drosophila melanogaster* Reared Axenically on a Fatty Acid–Free Holidic Diet

E.W. Rapport, D. Stanley-Samuelson, and R.H. Dadd

Division of Entomology and Parasitology, University of California, Berkeley (D.S.-S., R.H.D.), and Department of Zoology, University of Toronto, Ramsay Wright Zoological Laboratories, Toronto, Ontario, Canada (E.W.R.)

Wild-type *Drosophila melanogaster* were axenically raised on a completely synthetic fatty acid–free diet for at least ten consecutive generations, confirming that these insects do not require dietary polyunsaturated fatty acids. Capillary column gas-chromatographic analysis of lipids extracted from adults reared on yeast medium showed a peak which cochromatographed with linoleic acid, representing about 1.2% and 0.15% of all fatty acids in phospholipids and triacylglycerols, respectively. In flies reared on the synthetic diet for one generation or for five or more generations, the linoleic acid peak was still present but in about tenfold lower proportions of total fatty acids. This was true of both phospholipid and triglyceride fractions.

Key words: *Drosophila*, holidic diet, polyunsaturated fatty acids, sequential generation rearing

INTRODUCTION

An essential fatty acid requirement has been demonstrated for the majority of symbiote-free insects critically examined in this respect, predominantly Lepidoptera but including several Orthoptera, Coleoptera, and Hymenoptera [1]. In contrast, the many early nutritional studies of mosquitoes, houseflies, fleshflies, and fruitflies gave no indication of needs for dietary polyunsaturated fatty acids, suggesting that the Diptera as a group lacked this requirement. However, recent research now shows that a polyunsaturated fatty acid is dietarily essential for several species of mosquito [2]. The mosquito requirement is distinct from that of other insects in two ways: 1) It cannot be met by linoleic or linolenic acids, the C18 polyunsaturates that alleviate the deficiency in other insects, but is satisfied by various long-chain

Received January 16, 1984; accepted March 12, 1984.

Address reprint request to E.W. Rapport, Department of Zoology, Ramsay Wright Zoological Laboratories, 25 Harbord Street, Toronto, Ontario, Canada M5S 1A1.

polyunsaturates having structural affinities with arachidonic acid [3]; and 2) mosquitoes need only minute, vitamin-level dietary concentrations of arachidonic acid, in contrast to the 100-fold greater concentrations of linoleic or linolenic acids that are minimally necessary for other insects found to require essential fatty acid [4].

For most insects known to require an essential fatty acid, overt symptoms of deficiency appear late in development such as deformity at the adult molt, or diminished reproductive ability in the adult,or as poor development during a second generation of fatty acid deprivation. This, and the fact that most studies covered only one cycle of larval growth, prompted the suggestion that the seemingly general absence of a fatty acid requirement among the higher Diptera was perhaps more apparent than real [2]; it was argued that signs of deficiency might have emerged had rearings on fatty acid–free diets been carried through more than one generation, especially if, as for mosquitoes, only trace levels of fatty acids were in question. Trace levels of essential fatty acids could conceivably be provided in sufficient amounts for the needs of one larval growth cycle by reserves carried over in eggs from stock-reared mothers or as impurities in certain dietary ingredients. For example, although many species of mosquito are now known to require dietary arachidonic acid, symptoms of deficiency in *Aedes aegypti*, not detected in the many early studies, appear clearly as reduced adult viability, longevity, and egg production [1,5]. Also, although essential fatty acid deficiency in *Culex pipiens* reared on a completely defined synthetic diet is marked by the invariable failure of teneral adults to fly, adults reared on casein-based diets nominally lacking fatty acid often flew, presumably because the casein contained traces of active fatty acids.

We can find no example in the literature of a higher dipteran that has been reared through sequential generations on a synthetic diet devoid of materials such as casein, lecithin, or yeast RNA that might introduce covert dietary polyunsaturated fatty acids. Since one of us (E.R.) needed a defined diet that could be used to rear *Drosophila melanogaster* for studies of homeotic genes whose expression was recently found to be influenced by dietary manipulations of fatty acids and other nutrients involved in lipid metabolism [6,7], we wondered whether this fly could be reared indefinitely with a holidic medium lacking fatty acids or with such minimal and defined fatty acids as might prove essential for multigeneration development.

The extensive nutritional literature for *Drosophila*, recently reviewed by Sang [8], describes various amino acid–based diets approaching complete chemical definition and on which *D melanogaster* developed for one generation almost as well as on media containing yeast, its natural food. However, it is not clear that any of these formulations have been used with all the meridic components such as yeast RNA and lecithin (which would be most likely to contain lipid contaminants) replaced together by pure chemicals. And in no case were such diets reported to be suitable for sequential generation rearing. We therefore devised and tested a holidic medium of as completely chemically defined a composition as the purity of available chemicals allowed. Our initial expectation was that growth would be good for a first generation on synthetic diet, but, because an early study indicated some

component of ovolecithin other than its constituent choline or contaminant sterol improved first generation growth [9], we anticipated declining development in subsequent consecutive generations, which we hypothesized might be obviated by polyunsaturated fatty acids. The results will show that it was otherwise.

MATERIALS AND METHODS

The wild-type *Drosophila* stock used was obtained from Dr D. Fristrom, Department of Genetics, UC, Berkeley. A homozygous *bithorax*^{1} stock and a heterozygous *Contrabithorax*^{3} stock were obtained from the California Institute of Technology Stock Center.

The yeast diet with which the fatty acid–free synthetic diet was compared consisted of 42.2 g cane sugar, 20 g baker's yeast, and 15 g agar per 1,000 ml of media to which 15 ml of 10% methyl-p-hydroxybenzoate in 95% alcohol was added as a mold inhibitor.

The synthetic diet was modeled after diets of Sang, Geer, and Hunt [8,10,11], with amino acids, nucleotides, and choline substituted for casein, RNA, and lecithin respectively. Carnitine was included following the suggestion of Geer [12], and trace metals were also added. The diet composition is presented in Table 1. After generation 3 on the synthetic diet, agarose was extracted with ether or Folch's solution (chloroform: methanol, 2:1, v/v) to remove possible lipid contaminants. The cholesterol was checked to be free of lipid classes other than sterols by thin-layer chromatography. In preparing the diet, all components but agarose, cholesterol, and vitamins are dissolved in heated-distilled water. The pH is then adjusted to between 6.5 and 7 with KOH (5.6%). Next, the agarose is dissolved and added to the hot mixture, followed by a colloidal cholesterol suspension prepared from a hot alcohol solution to which water is quickly added (20 mg cholesterol:2 ml absolute ethanol:8 ml water). Finally, the vitamins (containing choline and carnitine) are added. After stirring and volume adjustment the diet is dispensed in 5-ml quantities into 13 × 93 mm shell vials or in 12-ml quantities into 45 × 90 mm snap cap vials (403), plugged with foam stoppers, and autoclaved. Biochemicals were obtained from Sigma (St Louis, MO) and agarose was obtained from Calbiochem (La Jolla, CA).

Surface sterilization of wild-type eggs was achieved by a brief treatment with 80% ethanol, brief vacuum application, and shaking in 0.3% methylbenzethonium chloride for 40 min followed by sterile water rinses [13]. Eggs from mutant strains were subjected to longer treatment with methylbenzethonium chloride (2.5–3 hr) with no shaking because preliminary attempts to sterilize with shaking always resulted in very poor hatch. Eggs were transferred to vials in a sterile Pasteur pipette, and cultures were reared in an incubator at 25°C, with relative humidity, maintained by pans of water, between 70 and 85%. After sterile cultures were established, succeeding generations were obtained either by transferring parents aseptically to a fresh container or by allowing adults to lay eggs on the old media and transferring a sample of eggs and new larvae with a sterile weighing spatula.

When sterile cultures of wild-type flies on yeast medium had been established, rearings on synthetic diet were initiated and continued for sequential

TABLE 1. Composition of Synthetic Dietary Medium for *Drosophila melanogaster*

Component[a]	mg/100 ml	Component	mg/100 ml
Agarose	1,000	Adenosine-2′(3′)-monophosphate	60
Sucrose	1,000	Guanosine-2′(3′)-monophosphate	40
Cholesterol, 99%+	20	Uridine-2′(3′)-monophosphate	40
Arginine	80	Cytidine-2′(3′)-monophosphate	40
Histidine	100	Thymidine	20
Isoleucine	300	$NaHCO_3$	100
Leucine	200	KH_2PO_4	71
Lysine HCl	190	$K_2HPO_43H_2O$	489
Methionine	80	$MgSO_47H_2O$	82
Phenylalanine	130	NaCl	4
Threonine	200	Ca gluconate	5
Tryptophan	50	Fe·Na EDTA[b]	2
Valine	280	Zn·Na_2 EDTA	2
Glutamic acid	540	Mn·Na_2 EDTA	2
Alanine	50	Cu·Na_2 EDTA	0.5
Aspartic acid	50	Thiamine HCl	0.2
Cysteine	50	Riboflavin	1.0
Glycine	50	Nicotinic acid	1.2
Proline	50	Ca pantothenate	1.6
Serine	50	Pyridoxine·HCl	0.25
Tyrosine	50	Folic acid	0.30
		DL-Carnitine-HCl	1.0
		Biotin	0.02
		Choline chloride	6.0

[a]All biochemicals from Sigma Inc, except agarose (Calbiochem), B vitamins (Nutritional Biochemicals, now ICN), sucrose (Mallinckrodt), and Ca gluconate (Squibb & Sons).
[b]Trace metals sequestrenes, from Geigy, Inc, are chelates with sodium ethylenediaminetetraacetate.

generations. For all these rearings, 20 small vials were set out per generation with ten eggs per vial; most vials remained sterile and, on average, about half the eggs developed to adults for all generations on synthetic diet. Observations on development times summarized in Table 2 were made on small vial cultures for both yeast and synthetic diets. At the fifth generation, flies were collected for weight determinations and lipid extraction. Since comparable data had not been taken at the start of the experiment for yeast diet or the earlier generations on synthetic diet, eggs from the fifth generation on synthetic diet were returned to yeast diet rearing in large vials with approximately 40 eggs per vial, to provide material for weight determination and lipid extraction. This was followed by transfer of eggs to synthetic diet to provide first generation flies for weight determination and lipid extraction.

Development time from egg to pupa was assessed as the time between the first egg seen in a container until the appearance of the first pupa; note that this value is not an average pupation time, but indicates the shortest time to pupation for any individual in the container. Samples of sexed flies were weighed in groups before lipid extraction for gas-liquid chromagraphic analysis occurred. Where gross microbial contamination was absent apparent

TABLE 2. Developmental Data for *Drosophila melanogaster* Reared on Yeast Medium or on Synthetic Medium for One and Five Generations

	Days to pupation		♀ Adult weight		♂ Adult weight	
Treatment	N[a]	Mean + SD[b]	N[c]	Mean + SD	N[c]	Mean + SD
Yeast medium	7	6.1 ± 0.38	12	1.13 ± 0.02	14	0.67 ± 0.04
Synthetic diet, 1st generation	7	8.7 ± 0.49[d]	6	0.62 ± 0.05	5	0.51 ± 0.03[d]
Synthetic diet, 5th generation	3	10.3 ± 1.15[e]	15	0.87 ± 0.08	16	0.53 ± 0.03[d]

[a]Number of containers observed. Value recorded was time between appearance of 1st egg and 1st pupa.
[b]SD = standard deviation.
[c]Number of samples weighed; samples contained 5–110 flies per sample, and for all mean values the numbers of flies exceeded 100.
[d]Significantly different from flies on yeast medium at the 0.05 level.
[e]Significantly different from flies of 1st generation on synthetic medium at the 0.05 level.

sterility was checked in all vials by innoculating brain-heart infusion broth (Difco, Detroit, MI). Contaminated fly cultures were discarded.

T-tests were used to determine if there were significant differences between means.

Lipids were extracted from flies in 2:1 chloroform/methanol (approximately 1 part flies to 19 parts extraction solution) and processed for lipid analysis, including purifying total lipids, separating lipid classes by thin-layer chromatography, transesterifying to methyl esters of the fatty acids, and preparing samples for injection into a gas-liquid chromatograph, as detailed elsewhere [14,15]. A 1-μl aliquot of each concentrated sample was injected into a Hewlett-Packard HP5792A chromatograph equipped with a SP-2350 fused silica capillary column (30 m, film thickness 0.20 μm; Supelco, Inc, Bellefonte, PA), a flame-ionization detector, and a HP-3390A recording integrator. Chromatograms were run isothermally at 195°C (helium carrier gas, 0.6 ml/min; split ratio about 1:30). Individual components, identified by comparison of retention times with single and mixed pure standards, were automatically quantified and reported by the HP-3390A.

RESULTS

At least ten consecutive generations of wild-type flies have been raised on a synthetic diet which lacks added fatty acids. Although data on development rate and adult weight were not taken for most of these generations, our impression was that after the initial generation of yeast medium rearing, second and subsequent generations on the synthetic medium were similar in developmental rate and adult size achieved.

To compare more precisely the effects of this diet to the yeast control diet, mean days to pupation and fly weights were determined for yeast medium, synthetic diet first-generation, and synthetic diet fifth-generation rearings. Progeny from flies grown on synthetic media for several generations developed significantly more slowly than those on synthetic diet for the first generation, and first-generation larvae developed significantly more slowly than those on yeast media (Table 2). Average weights for flies reared on the

different media are also given in Table 2. Females in all cases weighed more than males, but because female weight is probably greatly influenced by the amount of egg production, data on males are used to statistically compare diets. The yeast diet produced significantly heavier males than did the synthetic diet, and average weights for flies reared for the first versus fifth generations on synthetic diet were virtually identical.

To determine if polyunsaturated fatty acids are produced by flies and if this production varies with diet, gas-liquid chromatography studies were performed on flies raised on different diets. Lipids were extracted separately from males and females, but, because no differences in relative proportions of appropriate peaks were found, the data were pooled. A peak that cochromatographs with linoleic acid was found in most samples on both diets in phospholipid and triglyceride fractions. The relative proportions of this peak to total fatty acids are given in Table 3. Flies raised on yeast media had significantly higher proportions (an approximately tenfold difference) of the putative linoleic acid peak in both the phospholipid and triglyceride fractions than did those reared on synthetic diets. The fifth generation on synthetic diet contained the same relative amount of this fatty acid as those flies reared for one generation only. No other peaks representing polyunsaturates were found for either yeast or synthetic diet flies.

Two mutant stocks were reared on the synthetic diet, one containing the allele *bithorax*^{1} and the other *Contrabithorax*^{3}. Small numbers of eggs from both stocks consistently survived to adulthood, but these flies never reproduced.

DISCUSSION

The ability to rear *D melanogaster* axenically through ten sequential generations on completely defined dietary medium with no added lipids other than +99% pure cholesterol supports the view that this higher dipteran has no dietary essential fatty acid requirement. If this is indeed so, one of two propositions would seem to apply: 1) Polyunsaturated fatty acids are physiologically essential and in the absence of dietary intake can be biosynthesized; or 2) polyunsaturates play no physiological role for *D melanogaster* and are therefore neither required in the diet nor biosynthesized.

TABLE 3. Proportions of Linoleic Acid as Percentages of Total Fatty Acids in Tissue Lipids of *D melanogaster* Reared on Yeast Medium or on Synthetic Dietary Medium for One Generation and Five Sequential Generations

		Phospholipid % linoleic	Triacylglycerol % linoleic
Treatment	N[a]	Mean + SD[b]	Mean + SD
Yeast medium	14	1.24 ± 0.59	0.14 ± 0.10
Synthetic diet, 1st generation	5	0.08 ± 0.006[c]	0.02 ± 0.02[c]
Synthetic diet, 5th generation	8	0.12 ± 0.11[c]	0.06 ± 0.02[c]

[a]Number of lipid analyses.
[b]SD = standard deviation.
[c]Significantly different from yeast medium at the 0.05 level.

Our tissue fatty acid analyses aimed to clarify whether or not polyunsaturated fatty acids were biosynthesized. If tissue levels were maintained over several generations of synthetic diet rearing, during which time no exogenous intake could occur and during which any egg reserves of linoleic acid that might be carried over from mothers reared on the yeast medium would be diluted out, then polyunsaturates would have been synthesized steadily throughout the synthetic diet generations. In fact, no C20 or longer polyunsaturates were detected in this study, and the tenfold decrease in linoleic acid following transfer from yeast medium to one generation of rearing on synthetic diet indicates feeble or no ability to biosynthesize polyunsaturated fatty acid. On the other hand, no further diminution of the very small peak considered to be linoleic acid had occurred after a further four generations of synthetic diet rearing, which would indicate a continued biosynthesis, though very low, unless it is assumed either that a persistent unknown substance ran as a trace contaminant of the linoleic acid peak or that, in spite of our attempts to exclude dietary fatty acid contamination, traces of linoleic acid were in fact present in the diet, the most feasible source being perhaps the less than 1% of impurities in the cholesterol.

It has been an orthodoxy of animal nutritional metabolism that polyunsaturated fatty acids cannot be biosynthesized de novo, and this has been supported by a preponderance of evidence from insect studies [16,17] showing that neither linoleic nor linolenic acids are synthesized from precursors such as acetate, or derived from saturated or monoenoic acids, whether these latter are diet-derived or synthesized de novo. However, recent evidence now indicates that certain insects can synthesize linoleic acid [18], upsetting orthodox assumptions on this point. With respect to *D melanogaster,* it was concluded from tracer studies using labeled dietary acetate that no synthesis of linoleic or linolenic acids occurred [19]; nevertheless, a very small number of disintegrations above background were tabulated for these peaks, about 3% of disintegrations found for stearic acid and ca 0.5% of those for oleic and palmitic acids, all of which fatty acids are readily biosynthesized. Such trivial labeling was in the past disregarded as insignificant, but bearing in mind the exceedingly low essential fatty acid requirement of mosquitoes, even such trace labeling as recorded in Keith's study may now assume interest as a possible indicator of a minimal physiologically essential linoleic acid need.

If one chooses to ignore the persistent trace linoleic acid peak in our studies as artifactual in some way, or as a persistent contaminant, so that linoleic acid would be taken to be absent in flies reared on linoleic-free diet, then it must be supposed that this higher dipteran has no physiological need for any polyunsaturates whatever, either as a necessary structural component of lipid biomembranes or as a necessary precursor for the arachidonic acid route to prostaglandins (which latter materials have been detected in a not too distantly related dipteran, the housefly [20]). Since polyunsaturated fatty acids are considered normal components of animal biomembrane phospholipids in general, this would be a uniquely peculiar situation.

LITERATURE CITED

1. Dadd RH: Essential fatty acids for mosquitoes, other insects and vertebrates. In: Current Topics in Insect Endocrinoilogy and Nutrition. Bhaskaran GJ, Friedman S, Rodriguez J, eds. Plenum Press, New York pp 189–214 (1981).

2. Dadd RH: Essential fatty acids: Insects and vertebrates compared. In: Metabolic Aspects of Lipid Nutrition in Insects. Mittler TE, Dadd RH, eds. Westview Press, Boulder pp 107–147 (1983).
3. Dadd RH: Essential fatty acids for the mosquito *Culex pipiens*. J Nutr *110*, 1152 (1980).
4. Dadd RH, Kleinjan JE: Essential fatty acid for the mosquito *Culex pipiens*: Arachidonic acid. J Insect Physiol *25*, 495 (1979).
5. Sneller VP, Dadd RH: Lecithin in synthetic larval diet for *Aedes aegypti* improves larval and adult performance. Entomol Exp Appl *29*, 9 (1981).
6. Anastakis JD, Rapport EJ, Rogers I: Dietary fatty acid effects on morphogenesis in *bithorax* mutants. Arch Insect Biochem Physiol (in press).
7. Jowett T, Sang JH: Nutritional regulation of antennal/leg homeotic mutants in *Drosophila melanogaster*. Genet Res Camb *34*, 143 (1979).
8. Sang JH: The nutritional requirements of *Drosophila*. In: The Genetics and Biology of *Drosophila* 2a. Ashburner M, Wright TRF, eds. Academic Press, London (1978).
9. Sang JH: The quantitative nutritional requirements of *Drosophila melanogaster*. J Exp Biol *33*, 45 (1956).
10. Geer BW, Vovis GF: The effects of choline and related compounds on the growth and development of *Drosophila melanogaster*. J Exp Zool *158*, 223 (1965).
11. Hunt V: A qualitatively minimal amino acid diet for *Drosophila melanogaster*. Dros Inf Serv *45*, 179 (1970).
12. Geer BW, Dolph WW, Maguire JA, Dates RJ: The metabolism of dietary carnitine in *Drosophila melanogaster*. J Exp Zool *176*, 445 (1971).
13. Rapport E, Kleinjan J, Dadd R: Sterilization of *Drosophila* eggs without dechorionation. Dros Inf Serv *60* (in press).
14. Stanley-Samuelson DW, Dadd RH: Arachidonic and other tissue fatty acids of *Culex pipiens* reared with various concentrations of dietary arachidonic acid. J Insect Physiol *27*, 571 (1981).
15. Stanley-Samuelson DW, Dadd RH: Long-chain polyunsaturated fatty acids: Patterns of occurrence in insects. Insect Biochem *13*, 549 (1983).
16. Gilbert LI: Lipid metabolism and function in insects. Adv Insect Physiol *4*, 69 (1967).
17. Downer RGH: Functional role of lipids in insects In: Biochemistry of Insects. Rockstein M, ed. Academic Press, New York pp 57–92 (1978).
18. Blomquist GJ, Dwyer LA, Chu AJ, Ryan RO, de Renobles M: Biosynthesis of linoleic acid in a termite, cockroach and cricket. Insect Biochem *12*, 349 (1982).
19. Keith AD: Fatty acid metabolism in *Drosophila melanogaster*: Interaction between dietary fatty acids and de novo synthesis. Comp Biochem Physiol *21*, 587 (1967).
20. Wakayama EJ, Dillworth JW, Blomquist GJ: In vitro biosynthesis of prostaglandins in the reproductive tissues of the male housefly, *Musca domestica* L. Abstract 1010, Am Zool *20*, 904 (1980).