lations and years treated separately) revealed that the seasonal change in egg size was significant ($P < 0.05$) for LB 1989, LB 1990, and DP 1989, but not for DP 1990 ($P > 0.05$).

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20. The lower survival of enlarged hatchling males in most years would suggest that surgical effects on the female parent might cause a drop in offspring quality even though size remains enlarged. This is unlikely for the following reasons. First, we did not detect any effects of sham surgery ($P > 0.56$), follicle ablation ($P > 0.94$) (*5*), or yolk removal from eggs ($P < 0.47$) (*4*) except for the comparable effects that manipulations and natural variation have on egg size and offspring survival ($P < 0.01$) (ANCOVA with pooled data set). Second, the pathology would have to be sex-dependent (Fig. 2): the largest female hatchlings always showed high survival at the same time that the largest male hatchlings showed low survival. Third, on the first clutch in 1989 for LB, we did not release experimentally gigantized hatchlings, and thus the decreased survival of large hatchlings was a result of natural variation. Moreover, if we pool all results from both years and populations to estimate selection on the first-clutch male hatchlings with only the natural variation, we detected marginally significant optimizing selection ($P = 0.06$, $n = 240$). Fourth, in the most extreme case we observed directional selection that favored experimentally miniaturized male hatchlings (first clutch for LB 1990, Fig. 2A), and in this case significant directional selection favoring small males was also detected ($P < 0.01$) with only the natural range of variation.
21. We tested (ANCOVA) for differences in 1-month offspring survival as a function of egg size (covariate) across clutches (first versus later clutches), between populations, and between years (as well as higher order interaction terms among these three factors). We partitioned this analysis by sex because female survival curves were linear and male survival curves included a significant optimizing component. For the female offspring, we found that most of the higher order interaction terms involving clutch (for example, first versus later clutches) and the covariate egg size were significant or marginally significant, including clutch by year ($P < 0.05$), clutch by population ($P < 0.05$), clutch by year by egg size ($P = 0.06$), clutch by population by egg size ($P = 0.05$), clutch by population by year ($P < 0.01$), clutch by population by year by egg size ($P < 0.05$), as well as the term for the covariate egg size ($P < 0.05$). Thus, the intensity of directional selection on egg mass for female offspring varies across first versus later clutches, between populations, and between years. For the male offspring, a significant (egg mass)$^2$ term (optimizing selection) was used as a second covariate along with the covariate egg mass (directional selection). We found significant clutch ($P < 0.01$), egg size ($P < 0.01$), and (egg size)$^2$ ($P < 0.001$) effects. The only interaction term that was significant was clutch by egg size ($P < 0.01$). Population ($P > 0.05$) and year effects ($P > 0.05$) were not significant, nor were any other higher order interaction terms.
22. Postlaying body mass was used as a second covariate for the calculations of the directional selection coefficients on female fecundity, $m_e$. Significance levels based on jackknife estimates of $t$ values (*13*) for the directional ($\beta$) and stabilizing ($\gamma$) coefficients on fecundity ($m_e$) and offspring survival ($l_e$) (*15*) [where suggested by cubic spline analyses (*12*)] are as follows: LB 1989, first clutch: $l_e$ ($\beta = 2.686$, $P < 0.01$; $\gamma = -2.495$, $P < 0.05$), $m_e$ ($\beta = -0.618$, $P < 0.0001$); later clutches: $l_e$ ($\beta = 0.162$, $P = 0.05$), $m_e$ ($\beta = -0.397$, $P < 0.001$); 1990, first clutch: $l_e$ [$\beta = -0.007$, $P > 0.05$, not significant (NS)], $m_e$ ($\beta = -0.729$, $P < 0.0001$); later clutches: $l_e$ ($\beta = 1.175$, $P < 0.05$; $\gamma = -0.968$, $P < 0.06$), $m_e$ ($\beta = -0.556$, $P < 0.05$); and DP 1989, first clutch: $l_e$ ($\beta = 0.878$, $P > 0.05$, NS; $\gamma = -1.024$, $P > 0.05$, NS), $m_e$ ($\beta = -0.544$, $P < 0.0001$); later clutches: $l_e$ ($\beta = 0.177$, $P < 0.01$), $m_e$ ($\beta = -0.745$, $P < 0.001$); 1990, first clutch: $l_e$ ($\beta = 0.510$, $P > 0.05$, NS; $\gamma = -0.444$, $P > 0.05$, NS), $m_e$ ($\beta = -0.383$, $P < 0.05$); later clutches: $l_e$ ($\beta = 2.161$, $P < 0.05$; $\gamma = -2.191$, $P < 0.05$), $m_e$ ($\beta = -0.404$, $P < 0.0001$). The balance between fecundity and survival selection ($m_e \times l_e$) yielded an optimum egg size in seven of eight comparisons. The optimum egg size is the reproductive strategy that left the most surviving offspring. In one case (LB 1990, first clutch) there was no optimum per se, and the female that laid the smallest eggs in the population produced the most surviving offspring (arbitrarily defined as the optimum).
23. Significance levels based on jackknife estimates of $t$ values (*13*) for the directional ($\beta$) and stabilizing ($\gamma$) coefficients for survival selection (*15*) [where suggested by cubic spline analyses (*12*)] are as follows: LB 1989, first clutch (females: $\beta = 0.186$, $P = 0.05$; males: $\beta = 3.536$, $P < 0.02$; $\gamma = -3.361$, $P < 0.03$); second clutch (females: $\beta = 0.079$, $P > 0.05$, NS; males: $\beta = 1.196$, $P = 0.06$; $\gamma = -1.051$, $P = 0.09$); and 1990, first clutch (females: $\beta = 0.150$, $P < 0.02$; males: $\beta = -0.136$, $P = 0.06$); second clutch (females: $\beta = 0.307$, $P < 0.01$; males: $\beta = 1.353$, $P < 0.01$, $\gamma = -1.201$, $P < 0.05$). When both responses were linear (for example, first clutch of 1990), we could test for significant differences between the sexes in this case, selection on egg size differed significantly depending on offspring sex (ANCOVA, $P < 0.01$), and the probability of survival was highest for largest females but smallest males.
24. The curves relating survival probability to maturity are linear, which permits a test for differences in survival between the sexes by ANCOVA. Selection on egg size to maturity was significant (covariate egg mass was significant, $P < 0.01$), and there were significant sex differences in the strength and direction of selection on the survival of male and female offspring (sex by egg mass interaction term was significant, $P < 0.05$).
25. We thank R. Nichols, S. Edwards, and L. Carroll for assistance with collecting eggs and hatchling recaptures, P. Licht for research support (DCB-88-48022), and M. Wake for arranging support from the Department of Zoology, University of California (UC), Berkeley. This work was supported by a National Science Foundation grant to R.B.H. and B.S. (BSR-8919600); B S. was supported by the Miller Institute for Basic Research in Science, UC, Berkeley. We also thank P. Stadler for permission to work on his land. All animal care and surgery were in full compliance with the UC, Berkeley, animal care and use committee.

22 June 1992; accepted 20 October 1992

# Components of Sterol Biosynthesis Assembled on the Oxygen-Avid Hemoglobin of *Ascaris*

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**The parasitic nematode *Ascaris* infests a billion people worldwide. Much of its proliferative success is due to prodigious egg production, up to $10^6$ sterol-replete eggs per day. Sterol synthesis requires molecular oxygen for squalene epoxidation, yet oxygen is scarce in the intestinal folds the worms inhabit. *Ascaris* has an oxygen-avid hemoglobin in the perienteric fluid that bathes its reproductive organs. Purified hemoglobin contained tightly bound squalene and functioned as an NADPH-dependent, ferrihemoprotein reductase. All components of the squalene epoxidation reaction—squalene, oxygen, NADPH, and NADPH-dependent reductase—are assembled on the hemoglobin. This molecule may thus function in sterol biosynthesis.**

Ascariasis is an infection that pervades the world. The World Health Organization estimates that one billion people are infested with the etiologic parasitic helminth, *Ascaris lumbricoides* (*1*). Mortality rates for the disease are estimated at 20,000 per year, due mostly to biliary and intestinal obstructions. Morbidity is somewhat higher, with about one million cases per year exhibiting overt clinical manifestations (*2, 3*). Perhaps most devastating, ascariasis results in decreased growth and development in millions of affected children (*4, 5*). Little is known about the molecular metabolism of *Ascaris*. *Ascaris* appears to be microaerophilic, consistent with its location in the low-oxygen environment of the intestinal folds (*6*). Carbohydrate metabolism is anaerobic in *Ascaris*, but a terminal cytochrome chain exists that uses oxygen as available (*7*).

How oxygen is delivered to the cells remains unknown. *Ascaris* muscle has a myoglobin with high oxygen affinity (*8–11*), and the perienteric fluid has an abundant hemoglobin that binds oxygen 25,000 times more tightly than its mammalian homolog. (The partial pressure of oxygen at which the hemoglobin is half saturated is about 0.001 mm of mercury for *Ascaris* hemoglobin, and 25 mm of mercury for human hemoglobin.) (*8, 10, 12, 13*). The perienteric hemoglobin molecule is comprised of eight 40-kD subunits (*14*), but the structural features that promote its robust oxygen affinity are unknown. The function of this protein, which was detected spectroscopically by Keilin in 1925 (*19*) and characterized with respect to oxygen affinity by Davenport in 1949 (*8*), has also been a mystery. It binds oxygen too tightly to be

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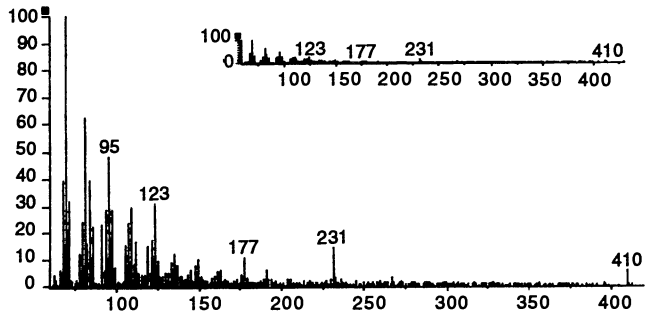


**Fig. 1.** Electron impact mass spectrometry. *Ascaris* hemoglobin was prepared by collection of hemolymph from *Ascaris suum* (Carolina Biological Supply Co.), ultracentrifugation at 75,000*g* for 20 hours to pellet the hemoglobin, and purification by HPLC on a Protein-Pak DEAE-5PW column (Waters) with a linear gradient of NaCl in 20 mM BisTris buffer (Sigma) at pH 7.0. This is a modification of an earlier procedure (*14*). The hemoglobin was further purified by chromatography on Protein-Pak SP-5PW (Waters) with a gradient of NaCl in 25 mM morpholine ethane sulfonic acid buffer (Fisher) at pH 6.0 followed by gel filtration on a Protein-Pak SW300 column (Waters) with phosphate-buffered saline as eluent. The hemoglobin, judged homogeneous by SDS-polyacrylamide gel electrophoresis analysis, was injected on a Nova-Pak C8 reverse phase column (Waters) and developed at 0.5 ml per minute with a linear gradient of acetonitrile from 10 to 100% in 0.1% trifluoroacetic acid. The unknown peak, eluting between 5 and 5.5 min, was collected and the electron impact mass spectrum was obtained with the use of a mass spectrometer (model ZAB SE) from VG linked to a VG model 11-250J data system. Shown is a typical spectrum. Authentic squalene was analyzed for comparison (inset). High-resolution analysis was also performed as described in the text.

used for delivery of oxygen to cells. It is more oxygen-avid than *Ascaris* myoglobin and, if it had access, would pull oxygen away from the muscle tissue (*8*, *15–18*).

We purified *Ascaris* hemoglobin to apparent homogeneity from perienteric fluid by ultracentrifugal sedimentation and then high-performance liquid chromatography (HPLC) sequentially on DEAE-cellulose, sulfopropyl (SP), and gel filtration matrices. To separate the globin from its associated heme, the purified protein was denatured in 10% acetonitrile, 0.1% trifluoroacetic acid and subjected to C8 reverse-phase HPLC. Three ultraviolet-absorbing peaks were noted corresponding to globin, heme, and an unknown. Electron impact mass spectrometry of the heme fraction revealed that it is identical to its mammalian counterpart, consistent with the possibility that *Ascaris* scavenges heme from its host (*17*).

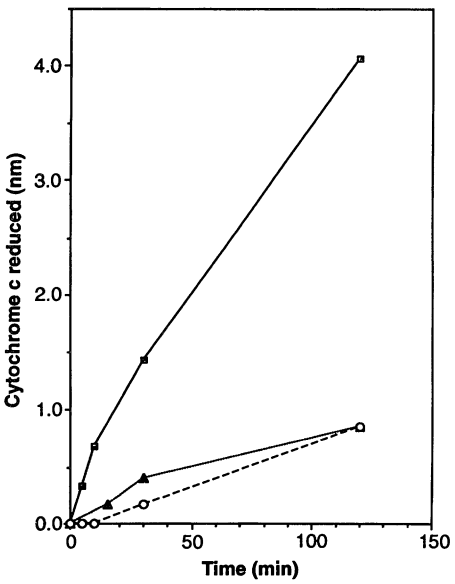


**Fig. 2.** Ferrihemoprotein reductase activity. Purified hemoglobin (0.06 mg) was incubated at 37°C with 100 μM NADPH and 20 μM oxidized horse heart cytochrome c (Boehringer-Mannheim) in 0.1 M $NaPO_4$, pH 6.1, in a final volume of 1 ml. The reduction of cytochrome c was followed spectrophotometrically at 550 nm. A control lacking NADPH and hemoglobin was subtracted from each. Squares represent incubation with NADPH and hemoglobin; triangles represent incubations with hemoglobin but no NADPH; circles represent incubations with NADPH but no hemoglobin. Values for cytochrome c reduced are in nanomoles.

The fraction containing the unknown compound was also analyzed by electron impact mass spectrometry (Fig. 1). High-resolution mass measurement of the molecular ion indicated a composition of $C_{30}H_{50}$ (calculated and measured mass 410.39). This composition and mass correspond to squalene. As confirmation, gas chromatography–mass spectrometry analysis showed identical retention times (25.50 min) and mass spectra (Fig. 1) for the unknown and for authentic squalene. Additionally, authentic squalene migrated at the identical position as the unknown upon reverse-phase HPLC.

During sterol biosynthesis, squalene is epoxidated and then cyclized to generate the sterol rings. In other eukaryotes, the epoxidation reaction requires molecular oxygen and an NADPH-dependent ferrihemoprotein reductase. Accordingly, we assayed our purified hemoglobin for intrinsic reductase activity (*20–22*). Hemoglobin was incubated with oxidized cytochrome c in the presence or absence of NADPH. Cytochrome c reduction was measured by spectrophotometry. *Ascaris* hemoglobin was capable of reducing cytochrome c in an NADPH-dependent manner (Fig. 2). The reaction was linear with time, enzyme concentration, and had a temperature optimum of 37°C with broad tolerance up to 50°C. The pH optimum was 6.0 to 6.5; perienteric fluid from which the hemoglobin was purified had a pH of 6.12. The reaction had Michaelis constants of 15.1 μM for cytochrome c and 17.4 μM for NADPH. A similar reaction rate was observed when NADH replaced NADPH. The ratio of reductase activity to hemoglobin protein was monitored throughout the purification to ensure that a partially co-purifying trace contaminant was not responsible for the reductase activity measured in the final preparation. Reductase-specific activity measured in nanomoles per hour per milligram of hemoglobin remained constant at each of the purification stages, and hemoglobin concentration paralleled reductase activity in each fraction of the final gel filtration chromatography step (Fig. 3).

That squalene copurifies with the hemoglobin through several steps, including gel filtration where it co-elutes at an apparent $M_r$ of 330,000, suggests that it is tightly bound to the hemoglobin molecule. Oxygen is also tightly bound (*8*, *10*, *12*, *13*). The demonstration that *Ascaris* hemoglobin has intrinsic NADPH-dependent ferri-

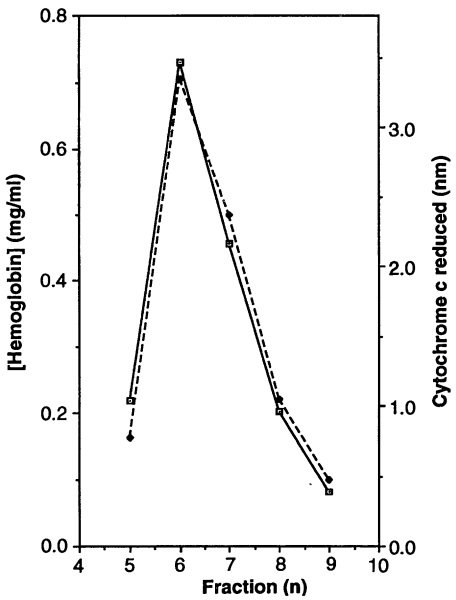


**Fig. 3.** Gel filtration profile of *Ascaris* hemoglobin. *Ascaris* hemoglobin was purified as in Fig. 1. The final gel filtration step was monitored spectrophotometrically at 410 nm to measure hemoglobin concentration, and each fraction was assayed for ability to reduce cytochrome c, as described in Fig. 2. Solid line represents hemoglobin concentration in milligrams per milliliter; dashed line represents nanomoles of cytochrome c reduced in a 1-hour incubation.

hemoprotein reductase activity therefore indicates that all the components necessary for squalene epoxidation are assembled on the hemoglobin molecule. It is not yet clear whether an additional oxidase is required for epoxidation, or whether this hemoglobin carries oxygen for use in the many other oxygen-requiring reactions involved in the conversion of squalene to cholesterol.

A clue to the physiologic role of this unusual molecule is the observation that female *Ascaris* worms have an order of magnitude more hemoglobin in their perienteric hemolymph than do males. Females each produce about 0.5 g of eggs (about 10% of total body weight) per day (*23*). The eggs are rich in sterols for use in the membranes of the developing larvae [sterols comprise approximately 2.4% of the dry weight of *Ascaris* eggs (*24*), comparable to the proportions found in ovine eggs (*25*)]. Thus, the female worms must synthesize large amounts of sterol, by a pathway that requires molecular oxygen, even though they live in the oxygen-poor intestinal folds. The parasites appear to have solved the problem by elaborating a hemoglobin that can sequester any available oxygen and channel it directly into cholesterol biosynthesis. Several related parasitic nematodes also have oxygen-avid hemoglobins (*22, 26*), which may therefore represent a special adaptation for intestinal helminths, or perhaps a more general feature of microaerophilic eukaryotes.

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27. The authors thank D Silbert, P. Ross, K. Bloch, and E. J. Corey for helpful discussions. Supported in part by a grant from the Lucille P. Markey Charitable Trust Mass spectrometry analyses were performed by F. F. Hsu at the Washington University Mass Spectrometry Facility supported by NIH grants RR-00954 and AM-20579

24 June 1992; accepted 30 September 1992


# Cryobiological Preservation of *Drosophila* Embryos


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**The inability to cryobiologically preserve the fruit fly *Drosophila melanogaster* has required that fly stocks be maintained by frequent transfer of adults. This method is costly in terms of time and can lead to loss of stocks. Traditional slow freezing methods do not succeed because the embryos are highly sensitive to chilling. With the procedures described here, 68 percent of precisely staged 15-hour Oregon R (wild-type) embryos hatch after vitrification at −205°C, and 40 percent of the resulting larvae develop into normal adult flies. These embryos are among the most complex organisms successfully preserved by cryobiology.**


Worldwide, some 10,000 to 20,000 lines of mutant *Drosophila* are maintained by frequent and costly transfer of breeding stocks. Such stock maintenance can result in genetic drift or the loss of stocks because of poor reproductive capacity, accident, mix-up, or contamination. Consequently, the ability to cryobiologically preserve such stocks indefinitely, as is done with stocks of other organisms such as the mouse and nematode *Caenorhabditis elegans*, would be of substantial value. A priori, one might consider embryos at the early stages better candidates for preservation than those at the later stages because of their simpler structure. We and Steponkus and co-workers found independently, however, that early embryonic stages of *Drosophila* were far poorer candidates for cryopreservation than 12- to 13-hour embryos, even though the latter contain about 50,000 cells (*1*) that are well differentiated into organ systems (hatching occurs at ~21 hours at 24°C). In contrast, mouse embryos are generally frozen at the 8-cell stage (*2*), and *C. elegans* larvae at the 558-cell stage (*3*).

The survival of cells exposed to cryogenic temperatures is critically dependent on (i) the avoidance of intracellular ice and (ii) the presence of intracellular molar concentrations of protective solutes. To fulfill these two criteria, cells must be permeable both to water and the cryoprotectant. Unfortunately, *Drosophila* are permeable to neither. The embryos are surrounded by a vitelline membrane that is rendered impermeable by waxes (*4*), and the larvae and adults possess a cuticle that is impregnated with lipids (*5*). To achieve cryobiological preservation, these barrier compounds must be removed without injury. Following the approach of Limbourg and Zalokar (*4*), Lynch *et al.* (*6*) and subsequently our group (*7*) developed methods for permeabilizing the vitelline membrane of 12- to 14-hour embryos. Our protocol exposes embryos for precise times (90 to 110 s) to heptane containing low (0.3 or 0.4%) and precise concentrations of alcohol (1-butanol). Our operational criterion of permeabilization is that the embryos stain ruby red or dark pink after 5 min in a 0.1% solution of the dye rhodamine B. The functional criteria are that they shrink in hyperosmotic (0.75 M) sucrose in D-20 *Drosophila* medium (*8*) in a few minutes (indicating permeability to water) and that they initially shrink and subsequently return to normal volume in about 20 min in 2 M solutions of ethylene glycol in D-20 (indicating permeability to that cryoprotective solute). On the basis of the staining criterion, the heptane-butanol procedure permeabilizes about 90% of 12-hour embryos. Over 80% of the embryos survive (hatch). This permeabilization procedure, however, is deleterious to 3-hour embryos.

The standard approach to successful


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