Heme Requirement for Reproduction of a Free-Living Nematode

Abstract. *The free-living hermaphroditic nematode* Caernorhabditis briggsae *has a nutritional requirement for heme. The organism can be subcultured repeatedly in a chemically defined axenic medium that contains autoclaved bacterial cells* (Escherichia coli) *and sterols if a hemeprotein-containing fraction from liver is present. Pure myoglobin, hemoglobin, cytochrome c, and hemin, respectively, can substitute effectively for the liver fraction.*

Several species of free-living nematodes have been serially cultured in axenic media (*1*). Reproduction of these organisms occurs only when the chemically defined medium (*2*) is supplemented with tissue extracts such as those from liver or chick embryos (*1*, *3*). Certain fractions of liver extract reportedly contain a single, biologically active protein (*4*) which possesses specific structural characteristics (*5*). However, this purified "growth factor" contains small amounts of lipids and nucleic acids in addition to protein (*4*, *6*).

We have observed that intact *Escherichia coli* will support the indefinite culture of the small, free-living nematode *Caenorhabditis briggsae* in a buffer-salt medium, providing that sterols are added (*7*). However, if the bacterial cells are first autoclaved, the nematodes will not reproduce, even in the presence of sterols and defined medium. Thus, some essential growth component in the cells is destroyed by heat. We have recently obtained a fraction from heated lamb liver extract which will substitute for this component. This material, obtained by chromatography of the extract on Sephadex G-100, yielded a red fraction (fraction A) which supported reproduction of the nematodes only if the defined medium contained autoclaved bacterial cells plus sterols (*8*). This liver fraction remains active after autoclaving for 8 minutes at 120°C. We now report that fraction A contains a hemeprotein and that the biological effect of this fraction can be duplicated by pure myoglobin, cytochrome c, hemoglobin, and hemin.

The heated liver extract was prepared by homogenizing approximately 50 g of lamb liver with an equal amount of water, by heating at 53°C for 6 minutes and by centrifuging at 39,000*g* for 30 minutes as described by Sayre *et al.* (*2*). The resulting supernatant solution is a deep crimson when fresh. The absorption spectrum of this solution is the same as that of the biologically active fraction A isolated from it. In both cases an absorption maximum occurs in the Soret region at about 415 nm, and satellite bands are present at 540 and 580 nm. The spectrum is characteristic of either oxymyoglobin or oxyhemoglobin. Based on the extinction coefficient at 418 nm, the concentration of hemeproteins in the original heated liver extract is approximately 3.0 mg/ml of extract or 6.0 mg/g (wet weight) of original liver.

There is evidence that the hemeprotein in fraction A and heated liver extract is myoglobin rather than hemoglobin or cytochrome c. Preparation of the extract at 53°C precipitates proteins such as hemoglobin to a large extent but not myoglobin (*9*); the chromatographic properties of fraction A on Sephadex correspond to those of a substance with a molecular weight similar to that of myoglobin rather than hemoglobin (*8*); fraction A is readily distinguished from cytochrome c by gel electrophoresis in a *p*H gradient.

The presence of hemeproteins suggested that this type of compound was implicated in the nematode "growth

Table 1. Assay of various supplements for their ability to support reproduction of *C. briggsae*. Compounds were added to a defined basal medium that contained sterols and autoclaved *E. coli* cells (see text). In experiment 1 the values in parentheses indicate the concentration of heated liver extract in percent by volume. Duplicate tubes containing 0.25 ml of medium were inoculated with three newly hatched larvae and incubated at 20°C. Larvae were observed daily and increments of growth were measured by an arbitrary grading system which was then converted to actual length. Generation time was estimated as the time at which the first newly hatched larvae appeared after the original organisms grew from a length of 200 μm to maturity. Final population was estimated after a period of 15 to 20 days. The population range is designated as + to +++++, the maximum number of worms per tube observed under the most favorable conditions. A population of ++ or more typically indicates that a second generation has appeared as a result of the maturation of the offspring of the original larvae.

Supplement	Concentration (μg/ml)	Growth rate (μm/day)	Generation time (days)	Final population
	Experiment 1			
Heated liver extract (53°C for 6 minutes)	0	140	n.r.*	
	5 (0.16%)	190	5.0†	+
	10 (0.31%)	200	3.9	++++
	20 (0.63%)	180	3.9	+++++
	Experiment 2			
Myoglobin (not autoclaved)	0	140	n.r.	
	25	200	6.3†	+
	50	190	3.5	++
	100	170	3.7	++++
	Experiment 3			
Myoglobin (autoclaved for 8 minutes at 120°C)	0	140	n.r.	
	12.5	100	n.r.	
	25	140	9–11	+
	50	190	3.2	++++
	Experiment 4			
Hemoglobin	0	160	n.r.	
	12.5	200	n.r.	
	25	270	2.6	++
	50	310	2.7	+++
	100	240	2.6	++++
Cytochrome c	25	260	2.7	+++
	50	240	3.1	++++
	100	230	3.3	+++++
	Experiment 5			
Cytochrome c	0	150	n.r.	
β-Lactoglobulin (not autoclaved)	100	140	n.r.	
β-Lactoglobulin (autoclaved)	100	80	n.r.	
Bovine serum albumin	100	110	n.r.	
Soluble casein (autoclaved)	100	80	n.r.	
	Experiment 6			
Hemin chloride	0	160	n.r.	
	1	170	7.7	+
	2	210	3.8	+
	4	210	3.1	++
	8	240	3.0	++++

* No reproduction. † Individuals in only one tube reproduced.

factor." The following information offers proof of this.

The assay of myoglobin and other materials for growth activity in *C. briggsae* is similar to that reported (*3, 7*). The chemically defined medium employed was originally reported by Sayre *et al.* (*2*) and modified by Buecher *et al.* (*10*). It consists of pure amino acids, glucose, water soluble vitamins, salts (including iron), and ribonucleotides (*11*). This mixture was further supplemented with sterols (50 μg/ml) in Tween 80 as described (*7*), and with autoclaved *E. coli* cells [0.3 mg/ml (dry weight)], which altogether comprise the basal medium. Axenic larvae of *C. briggsae* (usually three) were inoculated into a 10- by 75-mm culture tube that contained 0.25 ml of medium (in duplicate) and were incubated at 20°C. The effectiveness of the medium was determined by observing at intervals of 1 day or less the growth in length of larvae and reproduction of adult individuals.

In the basal medium alone, larvae grow to the size of advanced fourth stage larvae or small adults (650 to 850 μm long) in about 4 days, but they do not reproduce. As little as 0.31 percent (by volume) of heated liver extract (equivalent to 1.6 mg of original liver per milliliter of medium) supported extensive reproduction of the larvae (Table 1). Doubling the concentration of heated liver extract had little effect, whereas 0.16 percent proved to be inadequate.

As long as autoclaved bacterial cells are added, purified myoglobin (equine heart, Pierce Biochemicals, Rockford, Ill.) can replace heated liver extract as a growth requirement (Table 1). Myoglobin was dissolved in water at a concentration of 5 mg/ml, sterilized by membrane filtration (0.45 μm pore size, Millipore Filter Corp., Bedford, Mass.) and added to the above basal medium to give concentrations of myoglobin from 12.5 to 100 μg/ml.

Myoglobin becomes more effective for growth after autoclaving, although it does not effectively support growth at 12.5 μg/ml. The crude liver extract, at concentrations that support substantial growth, contained only 10 μg of hemeproteins per milliliter. However, autoclaved myoglobin at 100 μg/ml supports rapid reproduction and repeated subculture (ten serial transfers) of *C. briggsae* when it is substituted for heated liver extract.

A number of other proteins were tested in the same basal medium. Hemoglobin and cytochrome c were slightly more effective than myoglobin on a weight basis. Both compounds retained their activity after autoclaving. Non-hemeproteins such as β-lactoglobulin, bovine serum albumin, and soluble casein, each at a concentration of 100 μg/ml, were inactive (Table 1). The nutritional requirement for reproduction of *Caenorhabditis briggsae* under these conditions thus seems to be satisfied generally by hemeproteins, but not by protein in the absence of heme.

A question remains as to whether the active factor is the Fe-porphyrin moiety of the hemeprotein complex. A partial answer was obtained from experiments with hemin. Hemin chloride (Calbiochem, Los Angeles, Calif.) was dissolved in 0.1*N* potassium hydroxide, neutralized with hydrochloric acid to *p*H 7.8, diluted to 1 mg/ml, and sterilized by Millipore filtration in the same way as the myoglobin solution (Table 1).

At 8 μg/ml (equivalent to 200 μg of myoglobin on a molar basis), hemin was as effective as myoglobin at 100 μg/ml (Table 1); at 4 μg/ml hemin was approximately as effective as myoglobin at 50 μg/ml. At 2 μg of hemin per milliliter the generation time increased significantly and the total population was small. The 1 μg/ml concentration was ineffective. Therefore, on a molar basis, hemin is only about one-half as active as myoglobin although it is an effective substitute. One may speculate that the differences in effective doses could be related to problems of absorption; for example, precipitated versus soluble forms.

The results clearly demonstrate that hemin or heme compounds are required for reproduction of *C. briggsae* in a defined medium supplemented with sterols and autoclaved *E. coli* cells. Thus, one of the active components of liver extract (or of fractions derived from it) is most probably a hemeprotein. The meaning of the requirement for specialized structures reported by Sayre *et al.* (*5*) for their growth factor is unclear, especially since the material is replaceable by three contituents—a heme compound, a sterol, and a heat-stable component present in bacterial cells.

The requirement for heme may be related to the metabolism of hemeproteins in the parasitic nematode *Ascaris lumbricoides*. Smith and Lee (*12*) reported that the hemoglobin content of perienteric fluid increased when hemeproteins or porphyrins were added to the incubation medium; much hematin was incorporated into developing eggs of this organism.

W. F. Hieb
E. L. R. Stokstad
Department of Nutritional Sciences, University of California, Berkeley 94720
Morton Rothstein
Biology Department, State University of New York at Buffalo, Buffalo, New York 14214

References and Notes

1. M. Rothstein and W. L. Nicholas, in *Chemical Zoology*, M. Florkin and B. T. Scheer, Eds. (Academic Press, New York, 1969), vol. 3, p. 289.
2. F. W. Sayre, E. L. Hansen, E. A. Yarwood, *Exp. Parasitol.* **13**, 98 (1963).
3. E. C. Dougherty, E. L. Hansen, W. L. Nicholas, J. A. Mollett, E. A. Yarwood, *Ann. N.Y. Acad. Sci.* **77**, 176 (1959); W. L. Nicholas, E. C. Dougherty, E. L. Hansen, *ibid.*, p. 218.
4. F. W. Sayre, R. T. Lee, R. P. Sandman, G. Perez-Mendez, *Arch. Biochem. Biophys.* **118**, 58 (1967).
5. F. W. Sayre, M. C. Fishler, G. K. Humphreys, M. E. Jayko, *Biochim. Biophys. Acta* **160**, 63 (1968); F. W. Sayre, M. C. Fishler, M. E. Jayko, *ibid.*, p. 204.
6. W. F. Hieb and R. P. Sandman, *Fed. Proc.* **26**, 797 (1967); R. Pertel, thesis, University of California (1967).
7. W. F. Hieb and M. Rothstein, *Science* **160**, 778 (1968).
8. ———, *Arch. Biochem. Biophys.*, in press.
9. H. E. Snyder and J. C. Ayres, *J. Food Sci.* **26**, 469 (1961).
10. E. J. Buecher, E. Hansen, E. A. Yarwood, *Proc. Soc. Exp. Biol. Med.* **121**, 390 (1966).
11. Available as *C. briggsae* medium "75," Grand Island Biological Co., Grand Island, N.Y.
12. M. H. Smith and D. L. Lee, *Proc. Roy. Soc. London* **157B**, 234 (1963).
13. We thank Dr. W. D. Brown for his advice and assistance with the electrophoresis and absorption spectra. Supported in part by PHS grants AM 12625 and AI 07145.

24 November 1969; revised 5 January 1970

Pepsinogens: Genetic Polymorphism in Man

Abstract. *Population frequencies of the two patterns of pepsinogen excretion (with and without pepsinogen 5) and family studies indicate that pepsinogen 5 is controlled by a pair of genes,* Pg^a *and* Pg^b, *at a single autosomal locus and that* Pg^a *is dominant.*

We have demonstrated the existence of seven electrophoretically distinct pepsinogens (Pg 1–Pg 7) in extracts of human gastric mucosa, and have shown that they are clearly separable into two groups (*1, 2*). The five faster migrating zymogens, Pg 1–Pg 5, are limited to the fundus and body of the stomach and have been termed the group I pepsinogens. Pepsinogens 6 and 7 are also found in the antrum and proximal duodenum and have been designated the group II pepsinogens (*1, 2*). With the exception of Pg 1, the