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Biochemistry

Heme biosynthesis in bacterium-protozoon symbioses: Enzymic defects in host hemoflagellates and complemental role of their intracellular symbiotes

(uroporphyrinogen I synthase/isotope incorporation/hemin nutrition/trypanosomatid flagellates/bacterial symbiotes)

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ABSTRACT **Heme biosynthetic activity in the symbiotic association involving crithidial flagellates and intracellular bacteroids was studied by enzymic, nutritional, and isotope incorporation experiments. Component organisms and their complexes in this association were analyzed separately to determine the underlying cause of the hemin requirement of hemoflagellates and the role of symbiotes in sparing this requirement of two crithidial species.**

Nutritional study of symbiote-free flagellates showed that their growth requires at least 0.1 μg/ml of hemin, which can be substituted by protoporphyrin IX, but not by the porphyrin precursors, δ-amino-levulinic acid or porphobilinogen. These flagellates, in the presence of protoporphyrin IX, incorporated ^{59}Fe into heme, indicating that they possess ferrochelatase (EC 4.99.1.1), the terminal enzyme in the heme biosynthetic pathway, which catalyzes the insertion of iron into protoporphyrin IX.

In symbiote-containing flagellates serially cultured in a defined medium free of tetrapyrrole compounds, heme and porphyrins can be detected by a fluorophotometric method, indicative of heme biosynthesis. Study of [^{14}C]glycine incorporation into heme showed that the rate is much higher in symbiote-containing flagellates than in those without symbiotes. Microassay of uroporphyrinogen I synthase [EC 4.3.1.8; porphobilinogen ammonia-lyase (polymerizing)] revealed that the specific activity is high in symbiote-containing flagellates and higher still in isolated symbiotes, but essentially negligible in symbiote-free organisms.

It is concluded that the bacterial symbiotes augment a very limited heme biosynthetic capacity of host flagellates by supplying uroporphyrinogen I synthase and perhaps other enzymes preceding ferrochelatase in the heme biosynthetic chain.

Intracellular bacterium-like organisms are frequently associated with invertebrate animals, presumably cooperating to their mutual benefit (1). The precise nature of metabolic interchanges involved in such associations is obscure in most cases, although symbiotes often appear to have nutritional significance to their hosts (2). We demonstrate here a metabolic lesion of hemoflagellates in heme biosynthesis compensated for by the activity of their intracellular symbiotes.

A unique nutritional feature of hemoflagellates is their requirement *in vitro* for heme compounds, normally supplied to them as hemin, hematin, or hemoglobin (3, 4). Exceptional are several species of insect trypanosomatid flagellates, which, however, have been found invariably to harbor intracellular, self-reproducing entities (5–7), presumably symbiotic bacteria (5, 8, 9). In two of these species, *Blastocrithidia culicis* and *Crithidia oncopelti*, the prokaryotic nature of these entities has been recently confirmed. They resemble bacteria in ultrastructure, genome complexity, and sensitivity to antibiotics (10, 11, 14). Chloramphenicol treatment renders these flagellates permanently symbiote-free (12–15), but does not change the serological and DNA characteristics of the hosts (11, 14). Nutritional comparison of symbiote-containing and symbiote-free flagellates established that symbiotes spare hosts the requirements for hemin and for water-soluble growth factors present in liver extract (14, 15).

Of particular interest is the hemin-sparing effect of the bacterial symbiotes, which suggests that they endow the host flagellates with heme biosynthetic capability. It is generally presumed that the hemin requirement of hemoflagellates (naturally symbiote-free species) is correlated with their inability of heme biosynthesis, but supporting evidence for this has been fragmentary. On the basis of several criteria, we have investigated heme biosynthetic activities in symbiote-containing and symbiote-free flagellates, and in isolated symbiote fractions. The framework of our study is based on the general heme biosynthetic pathway outlined below: Glycine + succinyl-CoA → ALA → PBG → Uroporphyrinogen → Coproporphyrinogen → PROTO + Fe → Heme. Our findings not only elucidate symbiote-host interrelationships in heme biosynthesis but also define hemin nutrition of hemoflagellates in biochemical terms.

MATERIALS AND METHODS

Organisms and Cultures. Symbiote-containing strains of *B. culicis* and *C. oncopelti* were obtained from sources mentioned before (10). Symbiote-free strains of the two species were derived previously by chloramphenicol treatment (13–15). Naturally symbiote-free *Leishmania tarentolae* and *Trypanosoma conorhini* maintained in our laboratory were used for this study as reference species. Flagellates were cultured in defined or semi-defined media with or without hemin as described (10, 15).

Nutritional Studies. To determine the minimal hemin concentration for the growth of symbiote-free *B. culicis* and *C. oncopelti*, they were cultured in liver extract-fortified defined medium of Trager (16), to which different concentrations of hemin were added. Hemin in this medium was also substituted with heme metabolic intermediates, δ-aminolevulinic acid (ALA), porphobilinogen (PBG), or protoporphyrin IX (PROTO) at various concentrations to test for the growth of these flagellates. For these experiments, flagellates were inoculated into 2 ml of desired media at final cell density of 1×10^6 cells per ml and incubated at 27°. Growth, if any, was scored by cell count with a hemocytometer. Tests were considered positive only when flagellates

Abbreviations: Chemicals: ALA, δ-aminolevulinic acid; PBG, porphobilinogen; PROTO, protoporphyrin IX; URO-synthase, uroporphyrinogen I synthase. Organisms: BCA, symbiote-free *Blastocrithidia culicis*; BCN, symbiote-containing *B. culicis*; COA, symbiote-free *Crithidia oncopelti*; CON, symbiote-containing *C. oncopelti*.

could be successfully transferred at least six times in the same medium.

Heme and Porphyrin Assays. Symbiote-free and symbiote-containing flagellates were cultured in hemin-containing and hemin-free media, respectively. The latter medium was sometimes supplemented with different concentrations of chelated iron, e.g., ferric-EDTA, ferric citrate, or ammonium ferrous sulfate. Flagellates were harvested at mid-late logarithmic phase and washed three times by centrifugation at 4000 × *g* for 10 min in phosphate-buffered saline (pH 7.5). Intracellular symbiotes of *B. culicis* and *C. oncopelti* were isolated by a procedure reported elsewhere (11). Five μl of flagellate or symbiote suspensions in duplicate were mixed with 0.5 ml of 1 M perchloric acid/methanol (1:1, v/v) to determine porphyrin concentration fluorometrically (17). Another 5 μl of suspension in duplicate were mixed with 0.5 ml of 2 M oxalic acid and immediately heated for ½ hr at 100° to determine heme concentration fluorometrically (18). Hitachi Perkin-Elmer MPF-2A spectrophotometer was used to determine the concentrations of heme and porphyrins (19). Protein concentration was determined by the method of Lowry *et al.* (20), using bovine serum albumin as standard.

Enzyme Assay. Flagellates and symbiote fractions were assayed for the heme biosynthetic enzyme, uroporphyrinogen I synthase [EC 4.3.1.8; porphobilinogen ammonia-lyase (polymerizing)] by the method of Sassa *et al.* (21). Suspensions of organisms in 5-μl aliquots were disrupted by freeze-thawing (in a dry ice/acetone bath, three times) in 25 μl of 0.1 mM PBG in 0.1 M phosphate buffer (pH 7.4) and then incubated at 37° for 1 hr in dark. Specific enzyme activity was expressed as nmol of uroporphyrinogen/g of protein per hr at 37°.

Isotope Incorporation Experiment. Overall capacity of heme biosynthesis was studied in both symbiote-containing and symbiote-free *B. culicis* and *C. oncopelti*. Two μCi/ml of [2-^{14}C]glycine (specific activity 1.43 μCi/mol) was used as substrate to determine its incorporation into heme. After appropriate incubation, human hemoglobin solution was added as carrier and hemin was crystallized (22). Hemin crystals were then dissolved in 1.2 ml of pyridine, of which 0.2 ml was used to determine the recovery of heme by the pyridine hemochromogen method (23) and the remainder was dried on a planchette for radioactive determination in a gas flow counter (Nuclear Chicago) with a counting efficiency of 30%. The recovery of hemin was usually 60–65%. The radioactivity was corrected for the counting efficiency and was expressed as dpm/mg of protein. Control was prepared by adding [2-^{14}C]glycine directly into carrier hemoglobin solution which was then treated as described above. The ability of symbiote-free strains to incorporate ^{59}Fe into heme was also studied and compared in hemin- and PROTO-containing media. Flagellates were processed as above, except that ^{59}Fe citrate was used as substrate (25 μCi/ml, specific activity 21.9 Ci/g of iron) and that the radioactivity was determined in a Packard auto-gamma scintillation spectrometer. The procedure for isotope incorporation experiments is outlined schematically in Fig. 1.

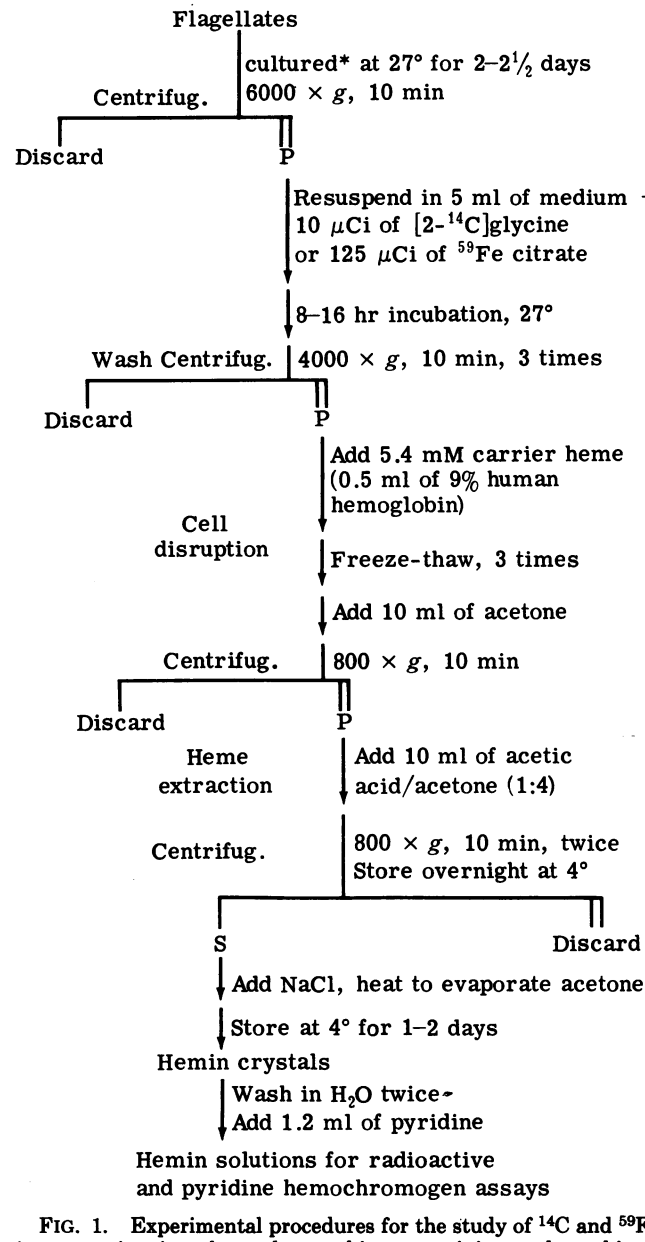

FIG. 1. Experimental procedures for the study of ^{14}C and ^{59}Fe incorporation into heme by symbiote-containing and symbiote-free *Blastocrithidia culicis* and *Crithidia oncopelti*.

*Semi-defined and defined media (4, 15), with and without hemin, were used for symbiote-free and symbiote-containing flagellates, respectively.

RESULTS

Nutritional Studies of Symbiote-Free Flagellates. Culture experiments, in which hemin was substituted with heme intermediates, revealed that symbiote-free *B. culicis* and *C. oncopelti* could grow in a medium containing PROTO, but not in the presence of ALA or PBG (Table 1). These results suggest that symbiote-free flagellates are able to synthesize heme by utilizing iron and PROTO, but not early porphyrin precursors. Successful serial culture of these flagellates was found to require at least 0.1 μg/ml of hemin in the culture medium (Table 1).

Heme and Porphyrins of Flagellates. By using a fluorophotometric method, heme and porphyrins were detected in both symbiote-containing and symbiote-free flagellates (Figs. 2 and 3). Since symbiote-free flagellates can be cultured only in media containing heme compounds, the amount detected probably represents that adhering to and/or absorbed by the organisms. On the other hand, the finding of heme and porphyrins in symbiote-containing flagellates is an indication of heme biosynthesis, since they have been cultured in media free of these substances. Symbiote-containing *C. oncopelti* appeared to have a high concentra-

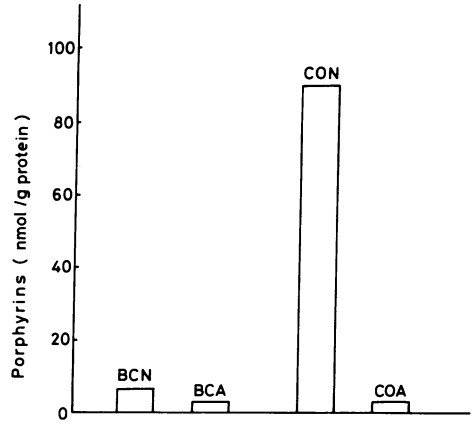

FIG. 2. Porphyrin contents of symbiote-containing and symbiote-free *B. culicis* and *C. oncopelti*. Porphyrins were extracted by perchloric acid/methanol mixture from flagellate pellets and were assayed fluorophotometrically. Culture media, here and below, were a chemically defined, tetrapyrrole-free medium for symbiote-containing flagellates and the same medium supplemented with hemin and liver extract for symbiote-free flagellates. BCA, symbiote-free *B. culicis*; BCN, symbiote-containing *B. culicis*; COA, symbiote-free *C. oncopelti*; CON, symbiote-containing *C. oncopelti*.

tion of porphyrins (Fig. 2) that was not affected by the addition of excess chelated iron to the media.

Uroporphyrinogen I Synthase of Flagellates. The specific activity of this enzyme was found to be 115–190 nmol of uroporphyrin/g of protein per hr at 37° in symbiote-containing flagellates, whereas little or no activity could be detected in symbiote-free flagellates, including *T. conorhini* and *L. tarentolae* (Fig. 4). In isolated symbiote fractions, however, this enzyme was found with specific activity 2 to 3-fold higher than that in the homogenate of symbiote-containing flagellates (Fig. 5).

Incorporation of ^{14}C and ^{59}Fe into Heme by Flagellates. Incorporation of ^{14}C from glycine into heme occurred in both strains of *B. culicis* and *C. oncopelti*, but the rate of incorporation was significantly greater for symbiote-containing flagellates than for those without symbiotes (Fig. 6). Thus, heme biosynthetic activity is higher when flagellates are associated with symbiotes. Despite the lower incorporation rate for symbiote-free flagellates, it does indicate a certain extent of heme biosynthetic activity, since essentially no radioactivity was detected in control preparations. Incorporation of ^{59}Fe into heme was studied only for symbiote-free flagellates. The incorporation rates were found to be 2 and 27 times higher in the presence of PROTO rather than hemin for *B. culicis* and *C. oncopelti*, respectively (Fig. 7). The isotope incorporation data are more significant in the case of *C. oncopelti* than *B. culicis*. It is uncertain whether this is due to the difference between the two species in the efficiency of substrate uptake or in the capacity of heme biosynthesis.

DISCUSSION

The minimal concentration of hemin supporting the growth of symbiote-free *B. culicis* and *C. oncopelti* was determined in this study to be 0.1 μg/ml—a value intermediate between those of *C. fasciculata* and *L. tarentolae* (3, 24, 25). As noted by others (24, 25), this value may not be necessarily the minimal requirement, since hemin tends to precipitate out in the culture media. It has long been recognized that hemin and related molecules may stimulate the growth of

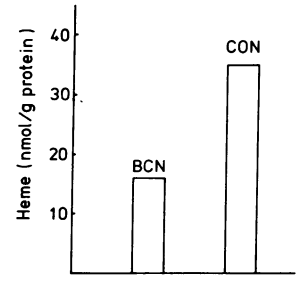

FIG. 3. Heme contents of symbiote-containing *B. culicis* and *C. oncopelti*. Heme concentrations were assayed by determining the difference of porphyrin concentrations before and after treatment of flagellates with 2 M oxalic acid at 100° for 30 min. Heme concentrations of symbiote-free flagellates were also assayed but were not shown here, as the culture media contained hemin, which rendered the determinations inaccurate.

certain unicellular microorganisms by serving a catalytic function, as iron carrier or the biosynthetic substrate which the recipient organisms cannot synthesize (26). Except for the artificial mutants of yeast and bacteria, organisms proven to fit the last category have been rare; these include a rickettsia (27), several species of bacteria (26), a fungus (28), nematodes (29), and possibly some obligate blood-sucking insects (3). The inability of these organisms to synthesize heme is mainly suggested by their requirement for minute amounts of hemin, which can often be substituted by PROTO, a molecule having neither iron nor catalytic activity. Nutritional studies of *C. fasciculata* (3) and of symbiote-free *B. culicis* and *C. oncopelti* in the present study indicate that they also meet these criteria. In addition, the latter are unable to utilize ALA and PBG, as is also true of *L. tarentolae* (25); and at least radioactive ALA has been shown to enter readily into hemoflagellates (25). Thus, these nutritional findings suggest that hemoflagellates may be generally incapable of adequate heme biosynthesis. The most signif-

Table 1. Growth of symbiote-free *Blastocrithidia culicis* and *Crithidia oncopelti* in semi-defined media* containing different concentrations of hemin or heme biosynthetic precursors

Compounds	Concentration† (μg/ml)	No. of experiments	Growth (× 10^{-6} cells/ml)	
			B. culicis	*C. oncopelti*
ALA	50—100	3	No‡	No
PBG	0.5—5	3	No	No
PROTO	25—50	7	44§	42
Hemin	25—40	17	37	40
	10	10	30	37
	1	10	24	31
	0.1	8	14	10
	0.05	6	No	No

* Trager's chemically defined medium containing 0.25% liver extract concentrate (4, 15).

† Final concentrations were adjusted by the addition of appropriate amount of stock solutions prepared as below: ALA, 5 mg/ml in Earle's balanced salt solution; PBG, 24.4 μg/ml in 0.1 M phosphate buffer at pH 7.4; PROTO, 1.7 mg/ml in 0.05 M NaOH; Hemin, 2 mg/ml in 0.05 M NaOH. Stock solutions were sterilized by filtration and stored at −20° in dark.

‡ No growth.

§ The mean peak population of serial culture experiments exclusive of the initial several transfers.

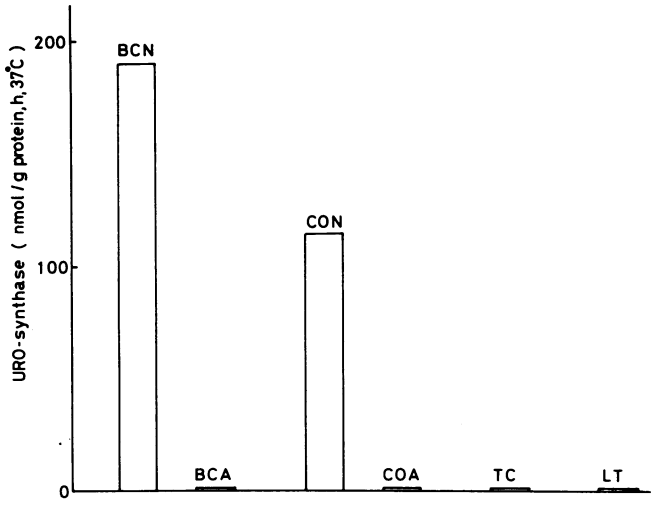

FIG. 4. Specific activities of uroporphyrinogen I synthase of symbiote-containing and symbiote-free *B. culicis* and *C. oncopelti*, *Leishmania tarentolae* (LT), and *Trypanosoma conorhini* (TC). See *text* for details of enzyme assay.

icant evidence for this is, however, provided in the present study by the finding of negligible activity of URO-synthase in symbiote-free *Blastocrithidia* and *Crithidia* as well as in *Trypanosoma* and *Leishmania*. Previously, similar evidence had been presented only for the hemin-requiring bacterium, *Hemophilus influenzae* (30). In consonance with the enzymic study, symbiote-free *B. culicis* and *C. oncopelti* appear to have limited ability to incorporate carbon from glycine into heme, as compared with symbiote-containing flagellates of the respective species. A comparable finding has been reported earlier by using [^{3}H]ALA as substrate for naturally symbiote-free *L. tarentolae* (25). Nutritional studies of hemoflagellates, which can be cultured in chemically defined media (4), have shown that they do not require the essential vitamin, B_{12}—a molecule sharing with heme the initial several steps in their biosynthetic pathways (31). Thus, hemoflagellates may have partial abilities for heme biosynthesis, which is demonstratable by the sensitive isotope experiments, although inadequate to support their growth. That symbiote-free flagellates are able to incorporate ^{59}Fe into

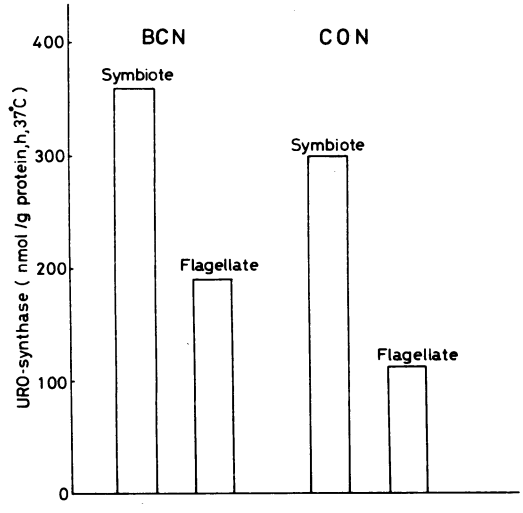

FIG. 5. Specific activities of uroporphyrinogen I synthase of symbiote-containing flagellates (flagellates) and symbiote fractions (symbiote) isolated from respective species of *B. culicis* and *C. oncopelti*. See *text* for details of the enzyme assay.

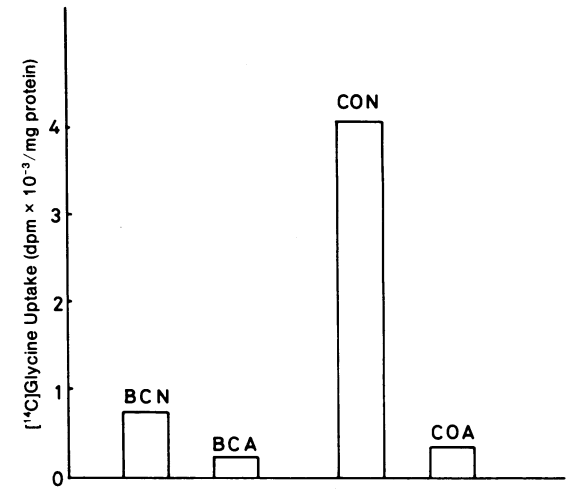

FIG. 6. Capacities of ^{14}C incorporation from glycine into heme by symbiote-containing and symbiote-free *B. culicis* and *C. oncopelti*. See Fig. 1 and *text* for experimental details.

heme confirms the nutritional finding that they can synthesize heme by utilizing PROTO and iron in the medium. This ability had also been found previously by nutritional experiments alone for *C. fasciculata* (3), but not for *L. tarentolae* (25). Thus, ferrochelatase, the enzyme inserting iron into PROTO (31), is functional in the former, but not in the latter. From our enzymic, nutritional, and isotope incorporation studies, it is concluded that symbiote-free *B. culicis* and *C. oncopelti* are deficient in URO-synthase and perhaps other enzymes preceding ferrochelatase for heme biosynthesis.

In the present study, high activity of heme biosynthesis was demonstrated in symbiote-containing flagellates which accounted for their lack of hemin requirement *in vitro* (15). Heme biosynthetic activity of these flagellates is indicated by the presence of porphyrins and heme in them when cultured in tetrapyrrole-free medium, of URO-synthase activity comparable to that of mammalian cells (21), and by the high incorporation rate of carbon from glycine into heme. Particularly noteworthy is the finding of substantial accumulation of porphyrins in symbiote-containing *C. oncopelti*—a phenomenon similar to certain types of clinical and chemically induced porphyria (32). The addition of excess chelated iron does not reduce this porphyrin accumulation,

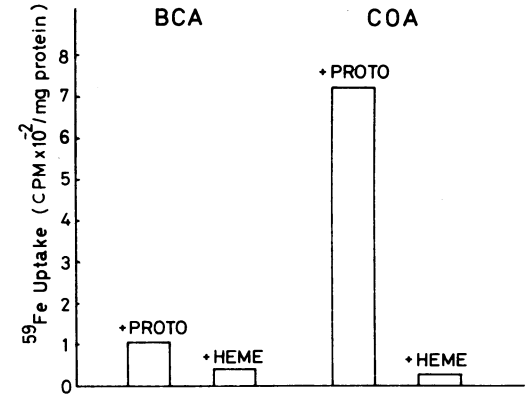

FIG. 7. Capacities of ^{59}Fe incorporation from iron citrate into heme by symbiote-containing and symbiote-free *B. culicis* and *C. oncopelti* cultured in medium containing hemin (+HEME) or protoporphyrin IX (+PROTO). See Fig. 1 and *text* for experimental details.

suggesting that it is not a result of iron deficiency. Perhaps this anomalous porphyrin metabolism is a manifestation of a slight incoordination in an otherwise totally harmonious symbiotic association.

The finding of adequate heme biosynthesis only in symbiote-containing flagellates indicates that this activity is associated with the bacterial symbiotes. Indeed, the specific activity of URO-synthase in isolated symbiotes was found to be higher than that in symbiote-containing flagellates. Moreover, the recovery of this enzyme in symbiote isolation accords well with that of symbiotes based on cell count (11). Evidently, URO-synthase is synthesized by the bacterial symbiotes to support heme biosynthesis in the host flagellates. Perhaps, like rhizobial bacteroids of legume nodules (33), the symbiotes of the flagellates possess the entire heme biosynthetic chain and thus supply their hosts with the final product, heme. Alternatively, symbiote and host may cooperate in making contributions complementary and indispensable to each other for heme biosynthesis. This would suggest that the symbiotes might function like mitochondria, which, in mammalian cells, possess certain heme biosynthetic enzymes, with others being in the cytosol (31). However, *B. culicis* and *C. oncopelti* possess, besides symbiotes, well-developed mitochondria, which may well be the loci of ferrochelatase, as are those of mammalian cells (31). On the other hand, symbiotes of the flagellates contain URO-synthase, a soluble enzyme normally present in the cytosol of mammalian cells (31). Thus, the symbiote-flagellate complexes described here would seem to have a most intriguing system of cellular compartmentation for heme biosynthesis. In such context, the bacterial symbiotes would be subjected to the regulation of their hosts and thus more like cell organelles than independent organisms. Intracellular bacterial symbiotes are indeed often refractory to culture *in vitro* (1), numerically restricted, and show certain structural and molecular similarities to mitochondria (11). There is as yet no evidence as to how the flagellates may regulate their symbiotes. However, in view of the accumulation of porphyrins in symbiote-containing *C. oncopelti*, the rate-limiting enzyme for heme biosynthesis in the symbiotic association may be ferrochelatase, which may be regulated by the host flagellates to the extent of controlling their symbiotes. It is hoped that investigation on this aspect not only may elucidate symbiote-host metabolic relationships further, but also may throw some light on regulation of cell organelles in general.

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