The influence of 'host release factor' on carbon release by zooxanthellae isolated from fed and starved *Aiptasia pallida* (Verrill)

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Abstract

Symbiotic dinoflagellates (zooxanthellae) typically respond to extracts of host tissue with enhanced release of short-term photosynthetic products. We examined this 'host release factor' (HRF) response using freshly isolated zooxanthellae of differing nutritional status. The nutritional status was manipulated by either feeding or starving the sea anemone *Aiptasia pallida* (Verrill). The release of fixed carbon from isolated zooxanthellae was measured using ^{14}C in 30 min experiments. Zooxanthellae in filtered seawater alone released approximately 5% of photosynthate irrespective of host feeding history. When we used a 10-kDa ultrafiltrate of *A. pallida* host tissue as a source of HRF, approximately 14% of photosynthate was released to the medium. This increased to over 25% for zooxanthellae from anemones starved for 29 days or more. The cell-specific photosynthetic rate declined with starvation in these filtrate experiments, but the decline was offset by the increased percentage release. Indeed, the total amount of released photosynthate remained unchanged, or even increased, as zooxanthellae became more nutrient deficient. Similar trends were also observed when zooxanthellae from *A. pallida* were incubated in a 3-kDa ultrafiltrate of the coral *Montastraea annularis*, suggesting that HRF in the different filtrates operated in a similar manner. Our results support the suggestion that HRF diverts surplus carbon away from storage compounds to translocated compounds such as glycerol.

Keywords: Carbon flux; Coral; Host release factor; Nutritional status; Photosynthesis; Sea anemone; Symbiosis; Zooxanthellae

1. Introduction

Symbioses between Anthozoa and intracellular dinoflagellates ('zooxanthellae') are common in nutrient-poor tropical seas. Zooxanthellae promote the conservation and recycling of essential nutrients such as nitrogen (Muscatine, 1980; Cook, 1983; Rahav et al., 1989) and supply photosynthetically-fixed carbon to the host. This carbon is principally translocated as glycerol (Muscatine, 1967; Trench, 1971a,b, 1974; Grant et al., 1997), plus glucose (Trench, 1971a,b; Ishikura et al., 1999) and perhaps lipid (Patton et al., 1983; Muscatine et al., 1994). It can support host respiration, growth and reproduction, or it may be lost from the association (Davies, 1984; Steen and

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Muscatine, 1984; Edmunds and Davies, 1986; McCloskey et al., 1994).

It is commonly observed that freshly isolated zooxanthellae (FIZ) release a substantial proportion of photosynthate in response to host tissue extracts but release little when incubated in seawater (Muscatine, 1967; Trench, 1971b; Sutton and Høegh-Guldberg, 1990). This suggests that carbon release is induced by one or more chemical stimuli ('host release factors'; HRF) present in the host's tissues. The nature of HRF is controversial. Effective compounds/molecules in preparations from different host species may vary with respect to thermal stability, specificity and molecular weight (Muscatine et al., 1972; Montaño, 1990; Sutton and Høegh-Guldberg, 1990; Davy, 1994; Gates et al., 1995). It has been suggested that HRF in tissue extracts of the coral *Pocillopora damicornis* and the sea anemone *Aiptasia pulchella* consists of a suite of free amino acids (Gates et al., 1995, 1999), with taurine in particular inducing substantial release from *A. pulchella* symbionts (Wang and Douglas, 1997). However, free amino acids appear not to be the HRF in extracts of the corals *Plesiastrea versipora* (Withers et al., 1998) and *Montastraea annularis* (Cook and Davy, 1999), though low molecular weight compounds are involved (< 1000 M_r) (Grant et al., 1997; Cook and Davy, submitted).

Initially it was thought that HRF may induce release by altering the permeability of the algal cell membrane (Muscatine, 1967; Trench, 1971b; Muscatine et al., 1972). However, it has also been suggested that HRF diverts photosynthetically-fixed carbon from energy stores to low molecular weight compounds that are more readily translocated (Trench, 1971b; Hinde, 1988). This hypothesis has been supported by experiments with the coral *Plesiastrea versipora*. *P. versipora* homogenate stimulates an increase in zooxanthella glycerol pools, concurrent with a partial inhibition of triglyceride and lipid accumulation (Sutton and Høegh-Guldberg, 1990; Grant et al., 1997). It is not clear, though, whether this arises from increased glycerol synthesis or from diversion of glycerol away from triglyceride synthesis. Certainly, given that ^{14}C-glycerol release can be detected within 30 min of labeling, it is unlikely that released glycerol originates from the hydrolysis of existing triglyceride stores.

Typically, starch and lipid accumulate in nutrient-limited free-living microalgae (Turpin, 1991). When the sea anemone *Aiptasia pallida* (Verrill) is not fed, its zooxanthellae become nitrogen-limited (Cook et al., 1988, 1992) and they also accumulate starch and lipid reserves (Muller-Parker et al., 1996). Thus, if HRF operates by diverting photosynthetic carbon from the synthesis of storage compounds, then nitrogen-limited zooxanthellae exposed to HRF should release more photosynthate than nitrogen-sufficient zooxanthellae. Since zooxanthellae tend to be nutrient-limited in hospite (that is, in the host) with respect to nitrogen (Muscatine et al., 1989; Cook et al., 1992 and 1994) and/or phosphorus (Jackson et al., 1989), this mechanism could maintain increased rates of carbon translocation in the intact symbiosis. However, it should be noted that HRF activity has yet to be demonstrated in hospite.

Here, we manipulate zooxanthellar nutrient status by feeding or starving *A. pallida* (Verrill) to test the hypothesis that as nutrient deficiency increases, so too does the response of isolated zooxanthellae to HRF. To test the generality of our results, we also employ HRF obtained from tissue of the reef coral *Montastraea annularis*.

2. Materials and methods

2.1. Experimental organisms

A clonal population (Bermuda strain) of the subtropical sea anemone *Aiptasia pallida* (Verrill) was cultured as described previously (Cook et al., 1988). Anemones were maintained in an incubator at 25°C and 55 μmol photons m^{-2} s^{-1} on a 12-h light/12-h dark cycle. Anemones were either kept in 0.45-μm-filtered seawater (FSW) and fed six times per week with *Artemia* sp. nauplii, or kept in 0.2-μm FSW and starved for between 7 and 114 days (after which their health deteriorated noticeably). Seawater was originally collected from 400 m offshore, near Port St. Lucie, Florida. The degree of nitrogen limitation was assessed, by measuring the effect of 20 μM NH_4Cl on the rate of zooxanthellar dark carbon fixation (Cook et al., 1992). The extent of phosphorus deficiency was not assessed.

Pieces of the coral *Montastraea annularis* were collected from a depth of 3 m at Admiral's Reef, Key Largo, Florida. These corals were processed within 24 h of collection.

2.2. Preparation of host tissue ultrafiltrate

We prepared ultrafiltrates of *A. pallida* and *M. annularis* tissue to provide samples with HRF activity that could be used to repeatedly assess the response of zooxanthellae over time. Well-fed specimens of *A. pallida* ($n = 18$) were homogenized in 0.2-μm FSW and centrifuged. The supernatant ('host fraction') was then fractionated by ultrafiltration at 4°C and $3000 \times g$, using 10-kDa pore-size Centriprep® concentrators (Amicon Inc.) and a Sorvall high-speed centrifuge. Coral tissue was removed from the skeleton using a recycling Water-pik® and 0.2-μm FSW, and an ultrafiltrate (3 kDa) of host tissue prepared as above. Both filtrates were stored at −17°C. The anemone filtrate was used within one month, while the coral filtrate was stored for 15 months before use. HRF activity was maintained during storage (cf. Fig. 1c, Fig. 2c), with both the 10 and 3-kDa filtrates containing all of the activity of crude homogenates (Cook and Davy, submitted; see also Grant et al., 1998).

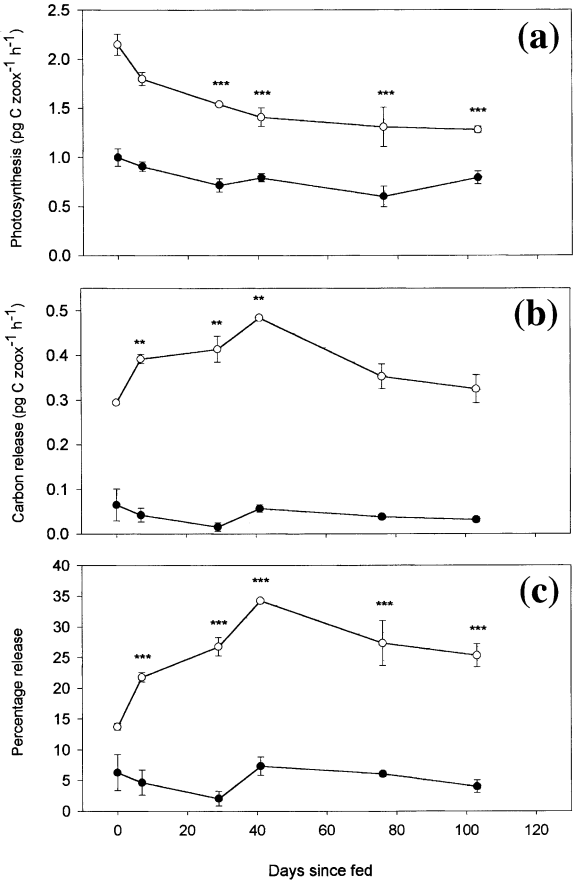

Fig. 1. The influence of increasing starvation on carbon fixation and release by freshly isolated zooxanthellae when incubated in a tissue extract of the sea anemone *Aiptasia pallida*. Zooxanthellae isolated from *A. pallida* and incubated in filtered seawater (FSW) or a 10-kDa ultrafiltrate of *A. pallida*. (a) Photosynthesis per zooxanthella vs. increasing starvation. (b) Total amount of carbon released per zooxanthella vs. increasing starvation. (c) Percentage of photosynthetically-fixed carbon released vs. increasing starvation. Values are means ± S.D., $n = 3$. Values that are significantly different from the 'well-fed' value are indicated by asterisks: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

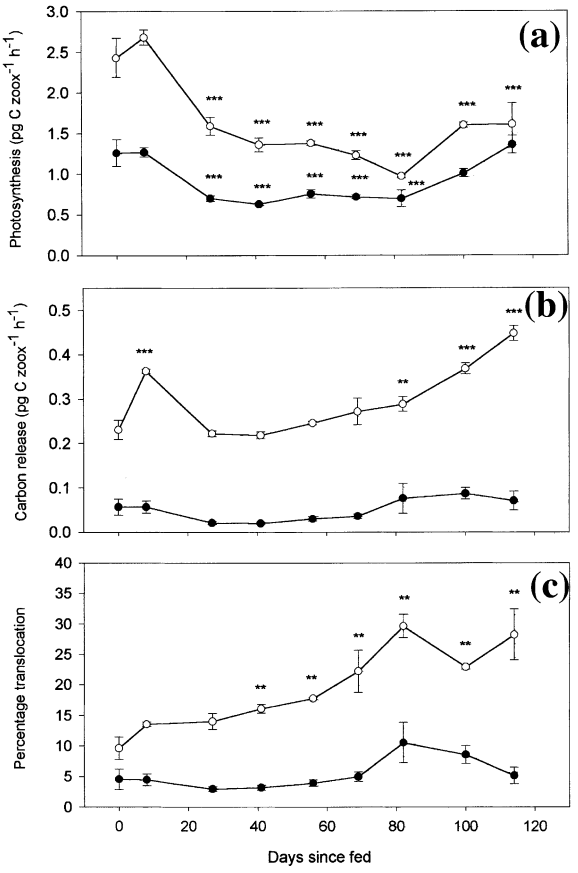

Fig. 2. The influence of increasing starvation on carbon fixation and release by freshly isolated zooxanthellae when incubated in a tissue extract of the coral *Montastraea annularis*. Zooxanthellae isolated from the sea anemone *Aiptasia pallida* and incubated in filtered seawater (FSW) or a 3-kDa ultrafiltrate of *M. annularis*. (a) Photosynthesis per zooxanthella vs. increasing starvation. (b) Total amount of carbon released per zooxanthella vs. increasing starvation. (c) Percentage of photosynthetically-fixed carbon released vs. increasing starvation. Values are means ± S.D., $n = 3$. Values that are significantly different from the 'well-fed' value are indicated by asterisks: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2.3. Measurement of carbon flux

Zooxanthellae were isolated from well-fed and starved specimens of *A. pallida*. Two to four anemones of the same nutritional history were homogenized together in FSW, and zooxanthellae isolated via centrifugation. The final algal pellet was re-suspended in FSW to give a cell concentration of $< 10^6$ cells ml^{-1}; release of carbon by zooxanthellae from *A. pallida* in response to host filtrate is linear up to this cell concentration (Cook, unpublished results). Cell concentration was determined using a haemacytometer.

One hundred microlitres of cell suspension was pipetted into microcentrifuge tubes, and 150 μl of either FSW ($n = 3$) or host filtrate ($n = 3$) was added to each. Three further tubes, containing 150 μl 20% formalin in FSW (saturated with sodium borate) and 100 μl of cell suspension, served as background controls. ^{14}C-bicarbonate (50 μl containing 2 μCi per tube; specific activity 56 μCi μmol^{-1}) was then added, the formalin tubes sampled for added activity, and all tubes placed on a light-table (cool-white fluorescent light; 250 μmol photons m^{-2} s^{-1}; 24.5 ± 0.5°C) for 30 min. The irradiance incident inside the tubes was sufficient to saturate photosynthesis. Tubes were agitated every 10 min to ensure that the zooxanthellae were in suspension.

Following incubation, 100 μl each of cells + medium and medium only (after centrifugation) were pipetted into scintillation vials. The contents of each vial were acidified to drive off unincorporated ^{14}C and the radioactivity determined with a liquid scintillation counter (Beckman LS-6500). Activities were corrected for background, and the photosynthetic rate, carbon release rate and percentage release calculated. This was done by using the mean specific activity (g C dpm^{-1}), which was estimated from the added activity and an assumed inorganic carbon content of 0.024 g C per litre of seawater.

All of the ^{14}C incubations were completed within a few days, with the filtrates being frozen when not in use. Also, zooxanthellae from one of the more recently fed groups of anemones were always tested simultaneously with zooxanthellae from one of the more extensively starved groups of anemones. This enabled any problems associated with filtrate age to be identified.

2.4. Statistical analysis

An angular transformation was used on all percentage data. Overall significant effects ($P < 0.05$) of starvation were identified by applying one-way ANOVA to either FSW or filtrate data. Subsequently, in those cases where the F-value indicated significance, Bonferroni pairwise comparisons were used to identify specific differences.

3. Results

3.1. Nitrogen status

The ratio of dark carbon fixation in 20 μM ammonium to that in seawater increased, from 1.3 to 2.0, between 2 and 61 days of starvation ($n = 2$ for each incubation). This trend is consistent with previously published data for *Aiptasia pallida* (Cook et al., 1992), and suggests increasing nitrogen deficiency with starvation. This is despite relatively high levels of inorganic nitrogen in the seawater: $[NH_4^+]$:1.3 ± 1.2 μM and $[NO_3^{2-}]$: 5.0 ± 1.6 μM (± S.D., $n = 4$).

3.2. Photosynthesis

Fig. 1a and Fig. 2a show photosynthetic rates (plus associated statistics) from the anemone and coral filtrate experiments, respectively. Both anemone and coral filtrates consistently enhanced photosynthesis over FSW rates in all samples, by a factor of 1.2–2.3 (cf. Trench, 1971a,b; Gates et al., 1995). The general trend in host filtrate was a significant decline in the photosynthetic rate with increasing starvation. When incubated with anemone filtrate, only FIZ from anemones starved for 7 days exhibited a photosynthetic rate similar to that of FIZ from the well-fed group. Indeed the rate of photosynthesis by FIZ from anemones starved for 29 days or more was only 72% or less of the rate by zooxanthellae from well-fed anemones. Likewise, when incubated in coral filtrate, only FIZ from anemones starved for 8 days exhibited a photosynthetic rate similar to that of FIZ from the well-fed group. The effect of starvation upon photosynthesis in FSW was less clear (Fig. 1a and Fig. 2a). For example, Fig. 2a shows an

initial decline, but no significant differences between zooxanthellae from anemones starved for 100 days or more and zooxanthellae from well-fed anemones.

3.3. Total carbon release

Total carbon release rates are shown in Fig. 1b (anemone filtrate) and Fig. 2b (coral filtrate). In both filtrates, the reduced photosynthetic rates of zooxanthellae from starved anemones were offset by high percent release values (Fig. 1c and Fig. 2c). For the anemone filtrate, zooxanthellae from hosts that had been starved for 7–41 days actually released significantly more carbon than those from well-fed anemones, while zooxanthellae from anemones starved for 76 days or more released similar amounts of carbon to zooxanthellae from well-fed anemones. In comparison, when incubated in coral filtrate, zooxanthellae from anemones starved for 100 days or more released significantly more carbon than zooxanthellae from well-fed anemones. There was no effect of starvation on total release of photosynthate by FIZ during incubations in FSW alone.

3.4. Percent release

Fig. 1c shows the effect of host feeding history on the percent release of photosynthetic products by zooxanthellae, when incubated in the anemone filtrate. Feeding history had no effect on percent release in FSW, which remained constant at approximately 5% of total fixed carbon. Incubation with the filtrate stimulated release over FSW rates in all cases and HRF activity was clearly retained over the experimental period. The percentage of photosynthate released in anemone filtrate increased significantly with starvation, rising from approximately 14 to $>25\%$. A similar pattern was observed when zooxanthellae were incubated in coral filtrate (Fig. 2c). The percent release increased from approximately 10 to $>30\%$ with starvation, although this change appeared to be more gradual than with the anemone filtrate; a significant effect was only observed after 40 days or more of starvation. As in the anemone filtrate experiment, there was no effect of host starvation on percent release in FSW.

4. Discussion

Our results demonstrate that, as freshly isolated zooxanthellae (FIZ) become more nutrient deficient, they release a greater percentage of their photosynthate when exposed to an extract of either sea anemone or coral tissue. This increased percent release offsets the decline in the cell-specific photosynthetic rate, so that the total amount of photosynthate released by zooxanthellae from starved anemones is similar to or greater than that released by zooxanthellae from well-fed anemones. We will discuss this observation in light of proposed carbon release mechanisms, and consider the possible ecological implications.

Previous models of nutrient fluxes in symbiosis have hypothesized that nutrient limitation increases the amount of carbon available for translocation by restricting algal growth (Muscatine et al., 1983; Falkowski et al., 1993; Dubinsky and Jokiel, 1994). In support of this model, the mitotic index of zooxanthellae in *Aiptasia pallida* declines from 8 to $<3\%$ during starvation (Cook et al., 1988), while lipid and starch stores of these cells increase (Muller-Parker et al., 1996). Using a light-regime comparable to that employed here, Muller-Parker et al. (1996) found that the cell volume occupied by lipid and starch in these algae doubled after four months of starvation. Nitrogen enrichment by the addition of ammonium has the opposite effect. When the coral *Pocillopora damicornis* was maintained in nitrogen-enriched seawater (50 μM ammonium) for 4 weeks, the size of its zooxanthella energy stores (primarily lipid) decreased by half (Berner and Izhaki, 1994). Starch accumulation by zooxanthellae in the giant clam *Tridacna maxima* was inversely related to the availability of nitrogen, supplied as 10 μM ammonium (Ambariyanto and Høegh-Guldberg, 1996); lipid levels were not determined in this study. In a similar fashion, translocation of fixed carbon in the coral *Porites astreoides* decreased with exposure to 10 μM ammonium (McGuire and Szmant, 1997). The most reasonable interpretation of these findings, and those of our experiments, is that carbon normally used for growth is diverted to the synthesis of energy stores because of a limited availability of nutrients. Nitrogen limitation of these algae (Cook et al., 1992) would be particularly important, as it would reduce the rate of synthesis of new proteins and nucleic acids.

However, starvation alone was not sufficient to enhance carbon release by our isolated zooxanthellae, as release was minimal in the presence of seawater alone, regardless of feeding history. It was apparent that increased release by zooxanthellae from starved anemones only occurred in the presence of host release factor (HRF). This is consistent with the HRF model proposed for the coral *Plesiastrea versipora*, where carbon is diverted from intracellular stores to the synthesis of compounds such as glycerol (Hinde, 1988; Sutton and Høegh-Guldberg, 1990; Grant et al., 1997). Moreover, the similarity of the response to both homologous and heterologous filtrates (from *A. pallida* and *Montastraea annularis*, respectively) could be indicative of a similar release mechanism in a number of host species. If HRF operates in hospite, then such commonality could be important if novel host-symbiont combinations arise, as could conceivably occur after coral bleaching events (Buddemeier and Fautin, 1993). However, the true generality of our results can only be ascertained by a more extensive study.

While photosynthesis decreased with starvation, perhaps due to reduced chlorophyll *a* levels and chloroplast volumes (Cook et al., 1988; Muller-Parker et al., 1996), this decrease was counterbalanced by the actions of HRF. Indeed, we have demonstrated that HRF activity interacts with nutrient-limitation to promote the release of photosynthate by isolated zooxanthellae. Such interaction could have important ecological consequences should it occur in hospite. In particular, corals dominate in nutrient-poor tropical seas, where the availability of nitrogen to zooxanthellae is limited (Høegh-Guldberg and Smith, 1989; Muscatine et al., 1989), leading to pronounced nitrogen-deficiency (Cook et al., 1994). Under these circumstances, zooxanthellae may readily translocate photosynthate to their host. In comparison, *A. pallida* is common in sub-tropical habitats that are relatively high in nutrients and food (Davy and Cook, personal observation), and its zooxanthellae show only moderate nitrogen deficiency under field conditions (Cook et al., 1992). Moreover, sea anemones tend to be voracious predators, while a number of zooxanthellate sea anemones are found at high latitudes where seawater nutrient levels are far in excess of those found in coral reef waters (reviewed by Muller-Parker and Davy, 2001). Consequently, these hosts may receive less photosynthetically-fixed carbon from their zooxanthellae than do reef corals. Certainly, the percent translocation values for zooxanthellae from well-fed *A. pallida* are very low when compared to other values in Fig. 1c, as well as various published values (e.g. Muscatine, 1967; Sutton and Høegh-Guldberg, 1990). It should be noted though, that factors other than nutrient availability, especially temperature and light regime, may also influence translocation rates in the field (Muller-Parker and Davy, 2001).

Of course, great caution should be exercised when relating the response of isolated zooxanthellae to the situation in the intact symbiosis. One point that should be stressed is that, at present, there is no direct evidence for the operation of HRF in intact symbioses. Vigorous homogenization, as was employed in this study, destroys the structure of host cells (Muscatine et al., 1994). It is therefore likely that the environment provided by a tissue extract is rather different from that provided by an intact host. In addition, typical HRF experiments may be complicated by physiological changes occurring to zooxanthellae upon isolation from the host (Goiran et al., 1997). The likelihood that some responses of FIZ are artefacts is suggested by our work with intact *A. pallida*. This has shown that the percentage of photosynthate translocated by zooxanthellae *in hospite* remains constant at approximately 16% when anemones are starved, under identical conditions to those used in the present study, for up to 3 months (Davy and Cook submitted; see Fig. 1c and Fig. 2c for contrast with FIZ). This could indicate that the regulation mechanism (HRF-related or otherwise), as well as other physiological events, differ between isolated zooxanthellae and those still inside the host. This point is also suggested by zooxanthellae from the giant clam *Tridacna crocea*, which predominantly release glycerol in isolation, but release glucose in the intact association (Ishikura et al., 1999). The resolution of this controversial matter will ultimately depend on the accurate identification of the HRF molecule(s) and the demonstration of its activity in hospite.

In summary, our results show that the nutrient status of zooxanthellae affects their ability to release short-term photosynthetic products during in vitro ('HRF') experiments, and indicate that nutrient status should be considered seriously in studies of HRF. The data are consistent with HRF-induced carbon release by isolated zooxan-

thellae, where carbon is diverted from intracellular stores to the synthesis of compounds such as glycerol (Hinde, 1988; Sutton and Høegh-Guldberg, 1990; Grant et al., 1997). However, data concerning the nature of storage pools, and the combined influence of nitrogen-supply and HRF upon these pools, are needed to confirm this interpretation. Likewise, our findings may have important implications for the ecological success of zooxanthellate invertebrates, but work on the operation of HRF in the intact symbiosis is essential before the true ecological importance of our findings can be determined. We therefore urge continued research on the character and operation of HRF.

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