Behavior, Hormones, and Hydra

Research on behavior of lower invertebrates may help elucidate some cellular actions of hormones.

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The line between behavior and physiology is tenuous. Although behavior is categorized by many scientists as an aspect of physiology, the distinction between them cannot be put off as simply a matter of semantics. The mere categorization of a phenomenon often determines how the problem will be investigated, and by whom—that is, what experimental approach will be taken, and whether the investigator will be a psychologist or a biochemist, an ethologist or a physiologist. In the case of the lower invertebrates, the demarcation between behavior and physiology becomes gossamer-thin because many lack well-defined nervous systems, endocrine systems, and structures usually associated with more highly organized forms.

Consider the implications arising from investigations of the feeding behavior of hydra or of some other coelenterates. Feeding in hydra consists of many separate steps: (i) a prey organism that accidentally encounters an outstretched tentacle is captured, wounded, and poisoned through the action of the deadly nematocysts (*1*) that line the tentacle; (ii) following capture of the prey the tentacles contract toward the mouth and the mouth opens; (iii) on contact with the mouth the food is ingested.

The second step (hereafter called the feeding response)—that is, the contraction of the tentacle toward the mouth and the opening of the mouth—is under chemical control. It has long been known that extracts of food elicit a feeding response in coelenterates (*2*, *3*). A landmark experiment was reported in 1955 by W. F. Loomis (*4*), who showed that the ubiquitous tripeptide reduced glutathione specifically activates the feeding response of *Hydra littoralis*. That specific biological substances of low molecular weight activate a feeding response has now been shown in a number of coelenterates (*5–11*).

In this article I discuss (i) evidence showing that reduced glutathione is an activator of feeding in *Hydra littoralis*; (ii) other coelenterates whose feeding responses are known to be activated either by glutathione or by other small molecules; (iii) a variety of behavioral responses of hydra that are either affected by or elicited by glutathione; (iv) studies on the mechanism of action of the glutathione receptor-effector system of hydra; (v) possible evolutionary relationships between chemical receptors of forms primitive in organization and hormone receptors of more complex organisms.

Reduced Glutathione and the Feeding Response of Hydra littoralis

The feeding reaction of hydra is accurately described by Ewer (*3*): "In this reaction the tentacles writhe and twist towards the mouth, while the mouth itself opens widely" (see Fig. 1). When Loomis (*4*) sought to identify the stimulator of feeding in *Hydra littoralis*, he used Ewer's criteria. By subjecting the food extracts to various treatments, Loomis (*4*) showed that the active principle in fresh tissue juice was heat-stable, active in small amounts, and labile both to long exposure to room temperature and to treatment with hydrogen peroxide. Fulton (*6*) repeated these experiments on hydra and obtained the same results.

Surmising that the active principle was a small oxidizable molecule, Loomis tested such biological compounds as ascorbic acid, coenzyme A, cysteine, and glutathione. Only reduced glutathione induced a feeding response. Concerned about the possibility that a trace amount of some substance might contaminate his glutathione preparation, Loomis obtained some chemically synthesized glutathione (*12*) free of biological contamination and found that it stimulated a feeding response.

Specificity for glutathione. To determine whether hydra responded only to the complete glutathione molecule, and not to any portion of the tripeptide, Loomis (*4*) tested compounds related to glutathione. He found γ-glutamylcysteine, cysteinylglycine, glycylcysteine, cysteine, and asparthione to be inactive. It is noteworthy that synthetic asparthione (β-aspartylcysteinylglycine) did not activate a response, and thus provided strong evidence of the specificity of the hydra receptor for glutathione. Asparthione has all the reactive groups of glutathione and differs from reduced glutathione only in that it lacks one methylene group. Among the various biological systems known to require glutathione, the feeding response of hydra is the only one not also stimulated by asparthione.

The unique specificity of the hydra receptor for glutathione was further documented by studies in which glutathione analogs and related amino acids were used (*13*, *14*). Data from all these investigations, summarized in Table 1, established the following. (i) The thiol is not required for activation; ophthalmic acid (γ-glutamyl-α-amino-*n*-butyrylglycine), norophthalmic acid (γ-glutamylalanylglycine), and *S*-methylglutathione also activated feeding. (ii) The hydra recognizes the specific structure of the intact tripeptide backbone of glutathione; this is evident because the analogs just mentioned activated feeding, and tripeptide analogs with large and charged substituents at the sulfhydryl grouping of glutathione competitively inhibited glutathione action. (iii) The receptor has a high affinity for the glutamyl part of the tripeptide; glutamic acid and glutamine were the only amino acids to show competitive inhibition. (iv) The α-amino of glutathione is probably required for the association of glutathione with the receptor; glutamic acid showed competitive inhibition, whereas α-ketoglutaric acid did not.

Knowledge of the inhibitory action of glutamic acid helped prove that reduced glutathione was the substance

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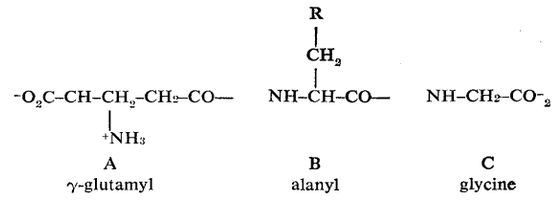
present in extracts of *Artemia salina* that elicited feeding. Addition of glutamic acid greatly decreased the activity of the extracts, while addition of glutathione to these extracts overcame the inhibition (*15*). Glutamic acid was not competing with ophthalmic acid in the extracts because this rare tripeptide, first found in calf lens (*16*), is not present in *Artemia salina* (*13*).

The weight of evidence regarding the specificity of the hydra receptor for glutathione was increased when Rushforth *et al.* (*17*) showed that the same conformation of glutathione plays another unique role, that of regulating two other receptor-effector systems in hydra, as discussed below.

My associates and I have also shown that, under rather special experimental conditions, such nontripeptides as trypsin (*18*) and zinc (*19*) can activate feeding responses. These findings do not contradict the results obtained with glutathione. On the contrary, they may provide us with tools for studying aspects of the mechanism of the glutathione-activated response (*20, 21*).

Controversy. Work by a few other investigators who claim that glutathione is not a specific activator of feeding in hydra has temporarily spiced the discussion of these phenomena. That Loomis' remarkable discovery (*4*) has evoked much excitement, new work, controversy, and confusion is not difficult to understand. He initiated the use of controlled conditions and precise analyses in a field of biology where major discoveries had been made without the need for such rigor. Nor is it surprising that his results contradicted those of prominent investigators, especially since his discovery concerned the biological role of a substance which acts in trace amounts. Modern biology is full of incidents in which work on trace substances, either as functional units (enzymes, hormones, vitamins, metals) or as contaminants, has caused confusion (*22*).

Table 1. Activators and inhibitors of the feeding response. The formula given below represents the basic tripeptide backbone of glutathione and its analogs. For example, when the R of the alanyl component (component B) is –SH, the formula represents reduced glutathione and, hence, is an activator. On the other hand, if R = –S–SG, then the formula represents oxidized glutathione, an inhibitor of the feeding response. [Adapted from Lenhoff (*15*).] A, B, and C refer to the three component amino acids of the tripeptide backbone of γ-glutamylalanylglycine, as follows:



Activators (tripeptide)	Inhibitors	
	Tripeptide	Others
R = –H	R = $-SO_2H$	Glutamic acid
R = $-CH_3$	R = $-SO_3H$	Glutamine
R = –SH	R = $-S-COCH_3$	Cysteinylglycine
R = $-S-CH_3$	R = –S(*N*-ethylsuccinimido)	
	R = –S–SG	
	R = –SH; A = $^-O_2C-CH-CH_2CO—$*	
	$^+NH_3$	

* In this special case A = β-aspartyl rather than γ-glutamyl.

Feeding Activators among Other Coelenterates

Loomis, in the last sentence of his 1955 paper (*4*), states, "The chemical mediator involved [in the feeding reactions of other coelenterates] may consist of glutathione in certain cases as in hydra, or may consist of some other cell constituent that functions in a similar manner."

If the procedures established by Loomis (*4*) and by Fulton (*6*) are followed, and if the precautions which I prescribed (*21*) are taken, a whole spectrum of amino acids, peptides, and possibly other substances may be found to function as specific activators of feeding in a correspondingly wide range of coelenterates. Some such compounds, recently found to be feeding activators, are discussed below.

Other hydra. Other species of hydra respond to glutathione, but not always in the same manner as *Hydra littoralis*. For example, *H. pirardi* may

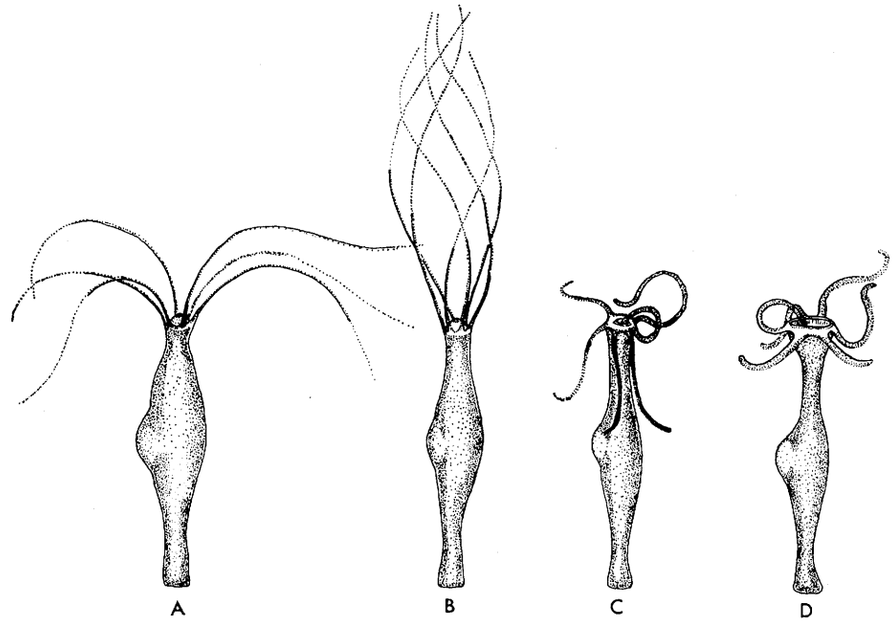


Fig. 1. Stages of the feeding response to reduced glutathione. (A) A hydra, in the absence of glutathione, is shown with its mouth closed and its tentacles outstretched and relatively motionless. (B) After glutathione is added the tentacles begin to writhe and sweep inward toward the animal's central vertical axis. (C) Next, the tentacles bend toward the mouth, and the mouth opens; shown in this composite drawing are the various positions that a tentacle takes before contracting. These movements, culminating in mouth opening, usually all take place within half a minute. (D) This drawing shows how a hydra looks during the greater portion of the feeding response, with its mouth open wide and the tentacles in various phases of contraction. Frequently, the tips of the tentacles are within the hydra's mouth, as shown in C and D. [Reprinted from the *Journal of General Physiology*, courtesy of Rockefeller Press]

respond to reduced glutathione for as long as 100 minutes at 22°C, closing and reopening its mouth many times during that period. In contrast, *H. littoralis* under similar conditions keeps its mouth open continuously for about 30 minutes. *Chlorohydra viridissima*, instead of opening its mouth wide in response to glutathione, as *H. littoralis* does, opens its mouth slightly, sometimes barely detectably, although it can ingest inert material immersed in glutathione (*23*). *Hydra pseudoligactis*, which responds to free glutathione, is observed occasionally to ingest inert material in the absence of added glutathione. Each species of hydra, therefore, may have its own peculiar feeding behavior (*24*). The results from experiments with *H. littoralis* should be used for purposes of comparison and should not be considered representative of all species of hydra.

Other glutathione responders. A dramatic response initiated by glutathione was exhibited by a marine hydrozoan, the siphonophore *Physalia physalis* (Portuguese man-of-war) (*5*). The man-of-war is a colonial coelenterate having numerous specialized zooids attached to a float. The function of food capture is carried out by one type of zooid [sometimes 9 to 12 meters (30 to 40 feet) long] (part of one of these tentacles is shown in the cover photograph) and the function of ingesting the food is carried out in a coordinated fashion by the feeding polyps, called gastrozooids. Figure 2 shows a detached single gastrozooid, one of the hundreds present in a large man-of-war. This single gastrozooid lay in the dish relatively motionless until the juice from a fish or a weak solution ($10^{-5}M$) of reduced glutathione was added. In response, the gastrozooid writhed, opened its mouth, twisted and turned until its lips attached to the glass container. The lips then began to spread as though attempting to engulf the dish. Figure 3 shows the gastrozooid after it had been in the glutathione solution for half an hour; the gastrozooid tube, 1 millimeter in diameter, has now become a disk more than 20 millimeters in diameter. Hundreds of gastrozooids pressing against the surface of a fish in a similar manner can envelop the fish, forming a complete "stomach" around it (*25*).

In another siphonophore, *Nanomia cara*, the writhing activities associated with feeding have also been ascribed to the influence of glutathione (*7*). The only other organism that I have seen

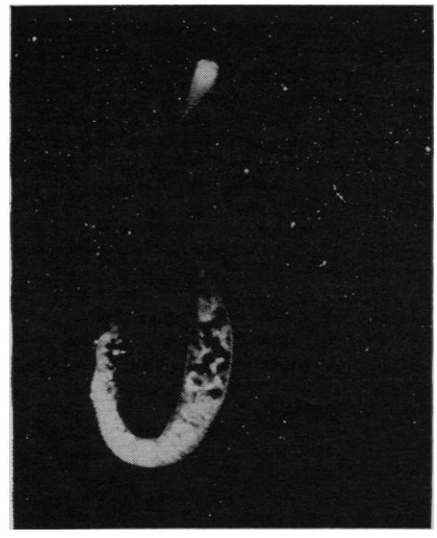


Fig. 2. Isolated *Physalia* gastrozooid. The mouth is the uppermost part at the end of the narrow cylindrical neck. [Reprinted from *Biological Bulletin*, with permission]

respond positively to glutathione is the calyptoblastic hydroid *Campanularia flexuosa* (*5*). In surveying more than 30 marine coelenterates at Woods Hole (Massachusetts), at Friday Harbor (Washington), at Coral Gables (Florida), and at Kaneohe (Hawaii), I did not see glutathione elicit a perceptible feeding response in any of the 30. Since most of these coelenterates gave a feeding response to extracts of *Artemia salina* nauplii, presumably they responded to compounds other than glutathione.

Proline responders. The first truly specific response to a specific chemical other than glutathione was shown by

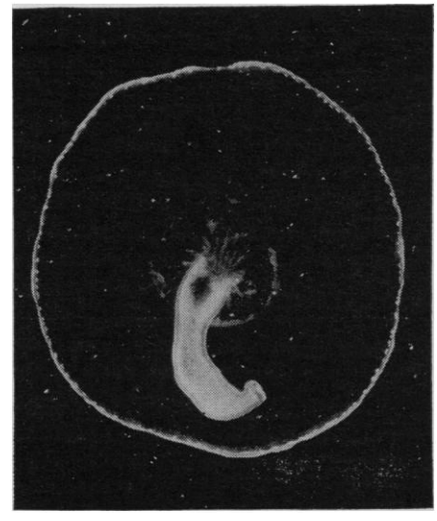


Fig. 3. A gastrozooid induced to spread by the presence of reduced glutathione. [Reprinted from *Biological Bulletin*, with permission]

Fulton (*6*), using the gymnoblastic colonial hydroid *Cordylophora lacustris*, which lives in brackish water. He identified proline as the activator of the feeding response by extending Loomis' procedures to include chromatographic separation of substances in the tissue extracts. Using proline analogs, he showed that the four-membered ring azetidine-2-carboxylic acid was almost as active as proline, whereas the six-membered ring analog pipecolic acid had about a tenth of the activity of proline. Inactive were pyrrolidine hydrochloride, pyrrole-2-carboxylic acid, 2-pyrrolidine-5-carboxylic acid, *N*-acetylproline, glycylproline, prolylglycine, hydroxyproline, thioproline, and sarcosine. Thus, the *Cordylophora* receptor can recognize specifically the imino region of a heterocyclic α-imino acid which is neither substituted nor unsaturated in such a way as to affect the imino acid group (*6*).

Another gymnoblastic hydroid, the marine *Pennaria tiarella*, also responded to proline (*9*) at concentrations as low as $10^{-6}M$. The proline analog pipecolic acid also elicited a response. No other substances tested, including glutathione, elicited a feeding response in this organism.

The third coelenterate shown to give an unequivocal feeding response to proline was not another gymnoblastic hydroid but the coral *Cyphastrea (10)*. This scleractinian responded to proline at concentrations of 10^{-7} to $10^{-3}M$ and to pipecolic acid at concentrations of 10^{-8} to $10^{-3}M$. *Cyphastrea* in particular was interesting in that it also gave a feeding response to $10^{-4}M$ reduced glutathione and to its analog *S*-methylglutathione. Hence, the results with *Cyphastrea* offer the first well-documented case of a coelenterate giving a feeding response to two different types of molecules, proline at low concentration and glutathione at higher concentrations.

In fact, we can never say for certain that a coelenterate gives a feeding response to only a single molecule (or its analogs) because it is virtually impossible to test all substances present in tissue extracts. It is possible, however, to show, through use of analogs and through competition experiments [as was done in the case of glutathione and *Hydra littoralis* (*15*)], that the substance under consideration is the major one in the tissue extracts tested that stimulates a feeding response.

Valine activation. The feeding re-

sponse of the Hawaiian swimming actinian *Boloceroides* sp. was recently shown to be controlled by the branched amino acid valine (*8*). Isoleucine, which is basically a valine having an ethyl group instead of one of the branched methyls, is an effective competitive inhibitor. On the other hand, leucine, identical to valine in all respects except that the branch point is separated from the α-carbon by an additional methylene group, is not effective either as an activator or as an inhibitor. Thus, *Boloceroides* can be said to have a receptor specific for an n-α-amino butyric acid with a branch point at the β-carbon.

An important observation made in the *Boloceroides* experiments was that valine did not cause wide mouth opening but, rather, caused the coelenterate to swallow an inert object. Undoubtedly other coelenterates give a distinctive feeding response to a specific chemical activator only in the presence of solid material. In fact, in those reported instances of coelenterates ingesting so-called inert solid objects in the absence of added chemical stimulator—as demonstrated, for example, with *Epiactis prolifera* (*5*)—there remains the strong possibility that traces of stimulating compounds were present on the object or in the water.

Glutamine responder. For the past few years we have been growing in the laboratory a clone of unidentified acontiate pedal-lacerating sea anemone isolated from a floating sargassum weed in Biscayne Bay, Florida (*26*). We showed that this anemone responds to glutamine, though neither glutathione, glutamic acid, nor asparagine would activate a response. Like *Boloceroides*, this anemone did not give an easily recognizable response to its feeding activator unless solid material was also present (*11*).

Other Actions of Glutathione on Hydra

To this point I have discussed the action of glutathione in initiating the tentacle-bending and mouth-opening phases of the feeding response in hydra. In the past 5 years, mainly through the efforts of N. Rushforth of Case Western Reserve University, glutathione has been shown to influence the physiology and behavior of hydra in five other measurable ways (Fig. 4). It was demonstrated that this tripeptide (i) increased the rate of the tentacle-waving "concerts," discussed below

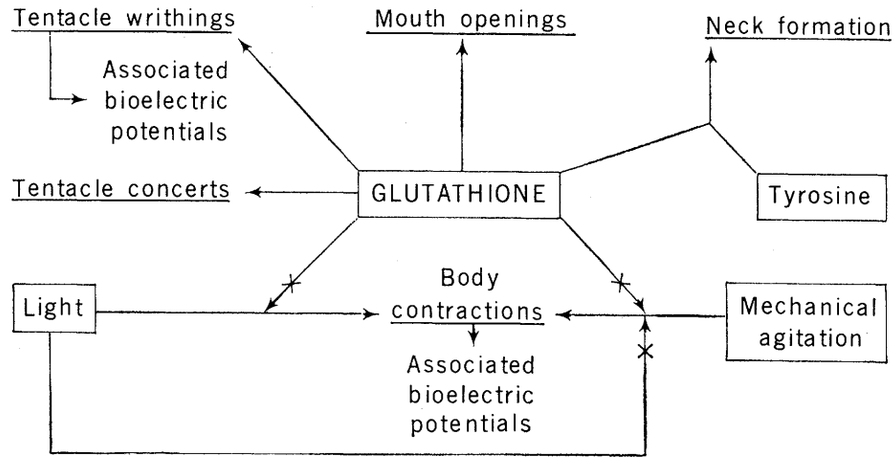


Fig. 4. Integration of receptor-effector systems. This diagram, which summarizes the various measurable effects stimulated by the action of glutathione on hydra, also emphasizes that the output of the glutathione receptor is linked with other receptor-effector systems of the animal. In addition, it points out an action of light in inhibiting the contraction response to mechanical agitation (*31*).

(*27*); (ii and iii) inhibited two behavioral contraction responses of hydra which are not part of the feeding response (*17*); (iv) stimulated changes in bioelectric potential (*28*); and (v) initiated a newly described behavioral response only when another chemoreceptor system was operating at the same time (*29*).

Tentacle concerts. The flexing motions of the tentacles sweeping inward toward the central vertical axis above the mouth are called tentacle concerts. These concerts occur spontaneously in hydra. Rushforth (*27*) finds that reduced glutathione in a concentration as low as $5 \times 10^{-10} M$ gives rise to a statistically significant increase in the frequency of tentacle concerts in *Hydra littoralis*, and that the frequency accelerates with increase in glutathione concentration up to $5 \times 10^{-9} M$. At this higher glutathione concentration, tentacle writhing commences. Tentacle concerts and tentacle writhing may be seen in Figs. 1B and 1C, respectively (*30*).

Glutathione inhibition of contraction responses. Rushforth (*17, 31*) has been conducting extensive and convincing experiments which show that the contractions of hydra induced either by light or by shaking, as well as the animals' spontaneous contractions, are inhibited while the animals are feeding on *Artemia salina*. Prompted by the experiments on the feeding response, Rushforth tested, first, *Artemia* extracts and then reduced glutathione; both inhibited the contractions. Using his quantitative procedures for measuring the inhibition of the contraction response, he showed (*31*) that the "modes of action of glutathione are similar to those discovered . . . [in] studying the mouth opening response."

In most of his experiments Rushforth used *Hydra pirardi*, European *H. viridis* (symbiotic and aposymbiotic), European stolonizing hydra, and *Chlorohydra viridissima* (Florida strain, 1961) (symbiotic and aposymbiotic). Such experiments demonstrate that many species of hydra have a specific glutathione receptor which not only controls feeding but, through its output, affects the contraction responses of the animal.

The mechanism by which glutathione inhibits the contraction response induced by light or mechanical agitation is unknown (*32*). Possibly there is a direct means whereby glutathione turns off the contraction responses, or glutathione may inhibit the response as an indirect consequence of having elicited the contractile events involved in the feeding behavior.

Electrophysiological correlates of glutathione-activated feeding response. Extending his research on the contraction responses, Rushforth found both indirect and direct electrophysiological correlates of the glutathione-activated feeding response (*28*). Just as homogenates of *Artemia* or solutions of glutathione inhibited the contraction response of hydra, so they inhibited the production of electrical potentials associated with either the spontaneous contractions of hydra or contractions induced by light (*33*). Furthermore, Rushforth found that the electrical potentials associated with the contrac-

tion of isolated tentacles of *Hydra pseudologactis* were not produced in the presence of $10^{-5}M$ reduced glutathione.

More striking is Rushforth's discovery that, when reduced glutathione inhibited the production of potentials associated with tentacle contraction, at the same time it directly initiated potentials associated with the glutathione-induced asymmetric tentacle movements. As the tentacle adapted to glutathione, the frequency with which these potentials were produced decreased, and the spontaneous tentacle contractions with their associated potentials were restored. Hence, these experiments not only present the first evidence of direct electrophysiological correlates of glutathione action but also provide strong evidence for the presence of glutathione receptor sites on hydra tentacles.

"Neck" formation. Neck formation was discovered by Blanquet and Lenhoff (*29*) using hydra (mostly *Chlorohydra viridissima* and *Hydra pirardi*) whose gastrovascular cavity was swollen with fluid and food particles (as observed between 1 to 6 hours following ingestion of food). Such hydra, when presented with *Artemia* extract or a solution of reduced glutathione, formed a tight constriction in the region just below the hypostome and sometimes extending over the adjacent one-third of the body tube. If, instead of a glutathione solution, the swollen hydra were presented with a live *Artemia* nauplius, the neck constriction formed, the mouth opened, and the hydra swallowed the nauplius. During ingestion the nauplius was carried down through the constriction, apparently by peristaltic contractions, and into the fluids of the swollen gastrovascular cavity. Hence, it would appear that these neck constrictions allow hydra to retain previously ingested food in the gut while swallowing newly captured prey.

Neck formation in *Hydra pirardi* was shown to be caused by a combination of three factors: (i) the presence of glutathione on the exterior of the hydra, (ii) distention of the wall of the hydra's body tube, and (iii) the presence of tyrosine within the gut. No other natural amino acid, including phenylalanine, could substitute for tyrosine. Analogs of tyrosine having blocked were inactive.

either the α-amino or α-carboxyl

From these experimental results we conclude that, in addition to the external glutathione receptor, hydra has an enteroreceptor specific for tyrosine. The hydroxyl, the α-amino group, and α-carboxyl group must all be present in order for the amino acid to be active (*29*).

The existence in hydra of two chemoreceptor systems that must act in harmony represents, to my knowledge, the first reported instance of two integrated, chemically mediated coordinating systems in the lower invertebrates.

Learning in hydra? During the past few years there has been renewed interest in the behavior of the lower invertebrates (*34*). One of the questions frequently asked is, Do they learn? I wonder if such a question is valid, especially with respect to organisms concerning whose behavior so little is known. Perhaps a better question would be, What is the behavior of such-and-such an organism, and how can we investigate it?

I have yet to be convinced that hydra can learn. Instead, it is becoming increasingly apparent that this animal has evolved many interacting receptor-effector systems (Fig. 4) that take care of its primary needs, most of which revolve around food and defense. To gain an understanding of hydra's behavior, therefore, effort should be directed toward understanding the properties, interactions, and mechanisms of its receptor-effector systems.

The Glutathione Receptor

Most information about the glutathione-elicited feeding response in *Hydra littoralis* came from studies in which this system was used as a model for the investigation of the mechanism of activation of a specific chemical receptor site. Such studies required animals that could respond to glutathione in a reliable and quantitative fashion. Of all the coelenterates investigated thus far, *H. littoralis* has proved to be the only one in which reliable quantification of the feeding response was possible.

With *Hydra littoralis* we were able to obtain animals that were genetically alike, in the same stage of development, derived from logarithmically growing culture, and grown in a defined environment (*30, 35*). Thus, there were always large numbers of animals that could respond to glutathione nearly synchronously.

Details of the procedures and of the present assay methods are given elsewhere (*30*). Suffice it to say that the major parameter of measurement was the "duration of the feeding response" —that is, the length of time the animal's mouth remained open in the presence of reduced glutathione. This assay, although a measure of behavioral response, is both accurate and objective because the investigator had merely to record the precise times that the hydra's mouth opened and closed. Since 1962 the assay always has been carried out at constant temperature and *p*H and in a solution of known ionic composition. The experimental hydra were placed directly into a solution of glutathione in order to activate all functioning glutathione receptor-effector systems and, thus, gain further control over the animals. These conditions differ, of course, from those in the pond, where hydra are presented with an oriented gradient of glutathione and of other substances emitted from the prey, in a solution of unknown composition. By controlling our experimental system in the manner described, we procured reproducible results with as few as five animals per measurement.

Studies aiming to uncover the mechanism of action of an excitatory substance can usually take many routes. One is the determination of the size and shape of the molecule that is active; this point is discussed above in regard to the excitatory effect of glutathione on hydra. In the rest of this section I deal with three other major questions: (i) What is the nature of the ionic media surrounding the receptor (and effector), and what are the effects of those ions on the response? (ii) What type of interaction occurs between activator and receptor? Is it fast? Is it slow? Is the activator metabolized? (iii) What are some of the properties of the receptor?

Studies of inorganic ions. Many inorganic ions affect the extent of the response activated by glutathione. Since these ions bathe both the receptor and the ectodermal effector cells, which are involved in part of the contractile processes of the feeding response, it is difficult to determine where and how these ions act. In any case, these studies are important because they define the limits within which the ionic composition can be varied, they reveal previously indiscernible aspects of the physiology of the animal, and they may add to our understanding of the mechanism of activation of the receptor.

Studies of the effect of environmental ions on the physiology of hydra are also of considerable importance in comparisons of work on hydra with work on other organisms. Hydras appear to be unique among members of the animal kingdom in that they are diploblastic, possess essentially no extracellular fluids (aside from the contents of the gastrovascular cavity), and live in fresh water. Thus the external medium plays a functional role similar to the *milieu intérieur* of higher forms. It is through this external environment that glutathione passes. Since external environments can be subjected to much more rigorous experimental control than the *milieu intérieur* can be, extensive study of environmental ions (*36*) should prove valuable.

Without calcium ions, hydra could not respond to reduced glutathione (*37*). The requirement for calcium was found to be *p*H-dependent (*38*), and a concentration of about $10^{-4}M$ was necessary for a maximum response. Strontium was the only ion that could substitute for calcium, and even it was much less effective (*37*). Magnesium ions were not required; in high concentrations they inhibited the responses by competing with calcium ions (*15, 37*). Sodium likewise competed with calcium, but less effectively than magnesium. The possible sites at which calcium can act in the complete glutathione receptor-effector system would seem to be innumerable.

Potassium ions were found to inhibit the feeding response (*20*), but, unlike magnesium and sodium ions, they did not act by competing with calcium ions. Concentrations of potassium ions as low as $10^{-4}M$ could lower the response to glutathione significantly, and this inhibition could be reversed by placing the animals in a potassium-free medium for a few hours (*38*). Since potassium ions play an important role in bioelectric potential, it may be that these ions act by affecting the cellular membrane potential of hydra.

The effect of such controllable environmental factors as anions, inhibitors, and temperature is described elsewhere (*15, 21, 37*).

Glutathione-receptor interaction. An indication of the speed at which the equilibrium between glutathione and the receptor was attained was determined by means of some relatively simple experiments. Hydra placed in a glutathione solution would open their mouths within a minute, and they would close their mouths within a minute after the glutathione was removed (*30*). These same animals could repeat this opening and closing sequence many times during an hour (*30*). Hence we can conclude (i) that glutathione has to be present constantly in the solution, and thus at the receptor site, in order for a response to take place, and (ii) that the equilibrium between glutathione and the receptor is rapidly attained.

Is glutathione consumed? The feeding response induced by glutathione has a finite period. This period is temperature-dependent—for example, about 30 minutes at 22°C (*15*). This limitation in duration is not caused by the disappearance of glutathione from the culture solution; that same solution of glutathione (or of the nonoxidizable analogs ophthalmic acid and *S*-methylglutathione), after removal from hydra that had made a maximum feeding response, induced a new group of animals to respond (*15*). Perhaps the cessation of the response was brought about by consumption of some substance in the receptor-effector system or, alternatively, by the production of an inhibitor. Whatever the correct explanation of this phenomenon, the hydra did not open their mouths in response to glutathione in the hour immediately following the end of a maximum response; in the subsequent 24 hours, however, they gradually regained their full capacity to respond (*15*).

The activation of a feeding response does not produce any detectable changes in the structure of reduced glutathione (*15, 30*). It is possible that changes may have occurred which were too small to be detected by present methods. In any case, it is not necessary to assume that glutathione is altered at all when causing a feeding response. There are known instances, as in enzyme induction by substrate analogs, in which a biological response is initiated by a molecule (noncoenzymic in function) combining with a specific site without that molecule being metabolized.

Behavioral determination of a dissociation constant. More recent experiments have centered around determination of the dissociation constant between glutathione and the receptor, and around use of the equilibrium data to elucidate the nature of the receptor site in the same way that an enzymologist uses data on K_M (the dissocation constant of the enzyme-substrate complex) to help determine the active site of an enzyme.

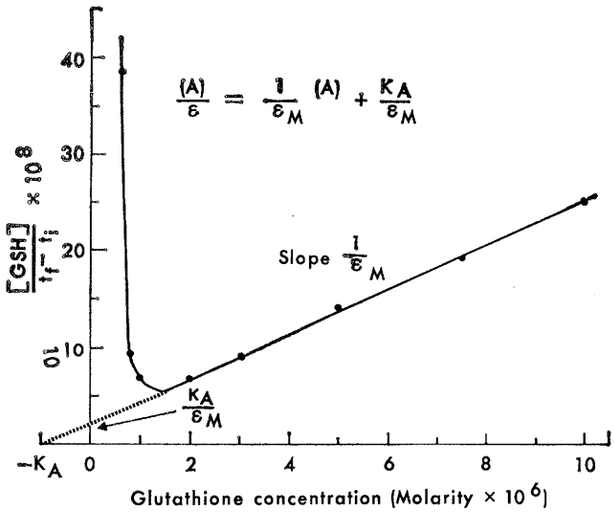
I have reported elsewhere (*21*) the assumption made in determining the dissociation constant, K_A, between the activator A and the receptor R. The effect of the activation is signified by $\mathcal{E}$, and the maximum effect, by $\mathcal{E}_M$. The equation derived,

$$\frac{(A)}{\mathcal{E}} = \frac{1}{\mathcal{E}_M}(A) + \frac{K_A}{\mathcal{E}_M}$$

is analogous to the second form of the Lineweaver-Burk (*39*) plot, the equation developed by Beidler (*40*) for mammalian taste chemoreception and, of course, a form of the Langmuir adsorption isotherm.

As shown in Fig. 5, a plot of $(A)/\mathcal{E}$ against (A) gives a straight line at most glutathione concentrations. The slope of the line is $1/\mathcal{E}_M$, and the extrapolated intersect is $K_A/\mathcal{E}_M$. If the line is further extrapolated, it intersects the abscissa at $-K_A$. Unlike data published according to the Lineweaver-Burk and Beidler plots, at low concentrations of glutathione the curve of Fig. 5 swings asymptotically upward. This upswing is always present and repeatable. It probably represents "below-threshold" activation. That is, at very low concentrations of glutathione the physiological response is not detectable by our behavioral assay; possibly the contractile fibrils involved in the animal's mouth-opening have not yet overcome the actions of those fibers that tend to keep the mouth closed. In the Lineweaver-Burk plot, such an upswing would be observed if the method used to assay the enzyme product were insufficiently sensitive. In the electrophysiological studies of Beidler, a similar upswing might result from plotting data at the "noise" level. The upswing of Fig. 5 gives promise of being a useful quantitative index of threshold. This upswing, however, is never seen at higher concentrations of glutathione; the line at higher concentrations of glutathione is straight and can be used to determine accurately the dissociation constant.

The dissociation constant of about $10^{-6}M$, as determined through a plot of this kind, is significant in at least four ways. (i) The smallness of the constant indicates a high affinity of the receptor for glutathione. (ii) Concentrations around $10^{-6}M$ are well within the physiological range to be expected under natural conditions of feeding. (iii) This constant provides a means of characterizing the receptor; that is, the glutathione receptor of *Hydra littoralis* may be said to



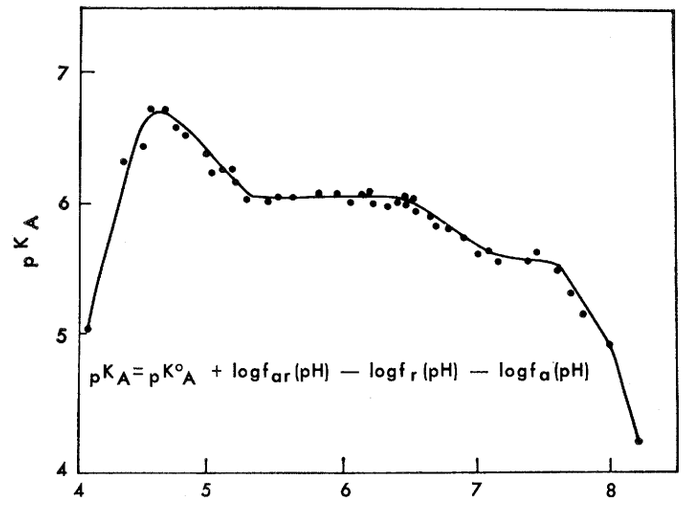


Fig. 5 (left). Plot for determining constants of the combination of glutathione with its receptor. GSH, reduced glutathione. [Reprinted from *American Zoologist*, with permission] Fig. 6 (right). Effect of *p*H on the dissociation constant K_A between glutathione and its receptor. [Reprinted from *American Zoologist*, with permission]

have a dissociation constant of $10^{-6}M$, under the given conditions. The constant is a characteristic of the receptor and remains nearly the same no matter what the nutritional state of the hydra (*31*). Similarly, experiments in which the buffer anion is varied alter the maximum response, but not the dissociation constant (*38, 41*). (iv) Changes in the K_A with *p*H can be used to determine the *pK*'s of the ionizable groups on glutathione or at the receptor site which are involved in the combination with glutathione.

The pH profile of the glutathione receptor. The *pK* measurements were made by means analogous to those used by enzymologists in determining the *pK*'s of ionizable groups at the active site of enzymes. For our purposes we needed an equilibrium equation, like Dixon's for enzymes (*42*), which would take into account the influence of *p*H on the dissociation constant. This modified equation (*20, 21*) involved the assumption that if the activator, receptor site, or activator-receptor complex ionizes, then, in the expression for equilibrium, each component (*A, R, AR*) equals its concentration multiplied by a term which is a function of *p*H. For example, if the activator ionized, then the total concentration of free activator, A_t, would be A times the *p*H function of A, *or* f_a (*p*H). The logarithmic form of the equation is:

$$pK_A = pK_A^0 + \log f_{ar}(pH) - \log f_r(pH) - \log f_a(pH)$$

Here pK_A refers to the negative logarithm of the dissociation constant of AR, while pK_A^0 is the same constant if none of the components has ionic groups; if none ionizes, then pK_A and pK_A^0 are equal. [The derivation of this equation is explained elsewhere (*21*).]

The foregoing equation, which indicates that a plot of pK_A against *p*H will consist of a series of straight lines joined by short curved parts, holds true for the glutathione-hydra system (Fig. 6). The results follow almost exactly the predictions from the modified Dixon equations. The following interpretations were made (*20, 21*). (i) Ionizable groups at the receptor site participated in binding glutathione, because significant variations in pK_A occurred with change in *p*H. (ii) The concave downward inflections at *p*H 4.6, 4.8, 6.5, and 7.6 represented *pK*'s of ionizable groups at the receptor site. These *pK*'s probably do not represent ionizable groups of glutathione, which have *pK*'s either below *p*H 4 (2.1 and 3.5) or above *p*H 8 (8.7 and 9.6) (*43*). If the receptor site is protein, then the determined *pK*'s may represent two β-carboxyls of peptide aspartic acid (or γ-carboxyls of peptide glutamic acid), an imidazole group, and a terminal α-amino group, respectively. (iii) The horizontal lines indicate *p*H values which do not affect the combination of glutathione with the receptor site. (iv) The quenching of the charges (see *42*) at around *p*H 4 and *p*H 8 indicated that receptor-site groups having *pK*'s of 4.6 and 7.6 may be associated with complementary charged groups on glutathione.

Proposed mechanism of binding and activation. A proposed mechanism for binding of glutathione to the receptor site suggests that the charged groups at the receptor site bind complementary charged groups on glutathione. This proposal takes into account previous data which show that the receptor recognizes the tripeptide backbone of glutathione and that the free α-amino of the glutamyl moiety of glutathione is implicated in binding to the receptor. Thus, the positively charged α-amino of glutathione might neutralize a negatively charged carboxyl of the receptor, while the terminal carboxyl of the glycyl moiety of glutathione might bind to a positively charged group of the receptor's terminal α-amino. Similarly, the groups represented by *pK*'s at *p*H 4.8 and 6.5 may be involved in the binding, or may be sufficiently close to the receptor site to be displaced somewhat during the binding process. These displacements are represented by the concave upward bends at *p*H 5.2 and 7.0 (Fig. 6).

The proposed binding mechanism points out the rigid specificity of the receptor for glutathione but does not tell us what happens after the combination occurs. Since, during activation, there was no detectable chemical alteration of glutathione and glutathione had to be constantly present at the receptor site, it was conjectured that glutathione operates by causing a reversible modification (possibly allosteric) of the tertiary structure of the receptor, which renders the receptor active (*15*).

Evolution of Chemical Receptor Sites

In animals as primitive in organization as hydra it is difficult to classify the glutathione-activated behavioral response of feeding in terms derived from extensive research done mostly with vertebrates and insects. Can we, for example, say that the actions of glutathione on hydra fall within accepted definitions for olfactory and gustatory phenomena? Are some of the actions of glutathione similar to those initiated by hormones or by hormone-like substances? Or must we revise or simplify our accepted definitions for chemical receptors to include the unique features of coelenterate feeding-activator systems?

Consider some of the characteristics and peculiarities of the glutathione-activated response. The activation of feeding takes place in response to the size and shape of a single molecule; hence, if we were to call the glutathione response a gustatory phenomenon, it would be a unique one in which no coding or filtering mechanism is involved—one which, instead, would consist of an all-or-none activation of a specific type of receptor site. Since glutathione courses through an aqueous solution, it is inappropriate to use terms reserved strictly for olfaction. Furthermore, when we think of an organism detecting a compound present in the environment, we do not usually envision that organism as playing an active role beforehand in releasing that compound from an environmental source. Yet such is the case with hydra. The glutathione response is a secondary behavioral event that normally occurs only after another receptor-effector system has been activated. During feeding, hydra does not even become exposed to glutathione until a small aquatic organism accidentally contacts the hydra's tentacles and the nematocysts in the tentacles discharge, puncturing the now captured prey (*44*). Then, and only then, is glutathione released from the prey into the environment.

The action of glutathione might be considered to represent, in hydra, a coordinating mechanism akin to some endocrine systems of higher metazoans, because glutathione initiates the coordination of a complex series of contractions and relaxations and because, like hormones, it acts in low concentrations [$10^{-9}M$ (*27*) to $10^{-7}M$ (*20*)]. This view was taken by Loomis in his original article (*4*), where he emphasized that glutathione starts the machinery which coordinates the manipulation of the captured prey for ingestion.

The chemical control systems of hydra also seem to be as thoroughly integrated with the physiology of the organism as the hormonal systems of higher forms are. Discovery of the tyrosine enteroreceptor (*29*) provides an example of a chemically mediated receptor-effector system that cannot exert its action unless another receptor, the glutathione one, is operating. Furthermore, Rushforth's demonstration (*17*) that glutathione inhibits the hydra's contraction responses to either light or mechanical stimulation shows the influence of one chemically mediated receptor-effector system over two other receptor-effector systems.

Yet it is apparent that we cannot call the glutathione response hormonal, because (i) the activator molecule comes from an organism of another species rather than from a gland within the organism itself; (ii) the fluid environment that transmits the glutathione is external rather than internal; (iii) the coordinated contractions are a visible response of the entire animal (hence termed behavorial) rather than a response of specific internal organs (hence termed physiological).

Were we looking for a name, glutathione, as it acts on hydra, might be called a "parahormone," a term "used to indicate conveniently those agents which act like hormones but do not entirely satisfy the accepted definition" (*45*). Some might even call it an "ectohormone." The danger of assigning a special label to a difficult-to-categorize phenomenon in a lower form of organism is that the name might overemphasize the unique aspects and hence obscure those features of chemical receptor mechanisms common to such systems in all metazoans. For example, despite the vast differences in complexity between hydra and mammals, the basic cellular events of receptor activation initiated by glutathione in hydra and by oxytocin in the uterus are probably similar. Both activators are relatively simple peptides that initiate coordinated responses in contractile tissue.

Thus we are left with the self-evident conclusion that, common to olfactory, gustatory, and hormonal (including ectohormonal) phenomena, is the following series of fundamental events: the combination of a cellular receptor with a specific molecule, leading to the initiation of a coordinated series of biological processes. Viewed in this light, the mechanism of activation is independent of such factors as whether the activating molecule issues from the prey or from an endocrine gland; whether the molecule traverses the external aqueous environment or the internal blood stream; whether the response is a behavioral contraction, an electrophysiological signal, or a sequence of developmental changes; or whether the phenomenon is classified as olfactory, gustatory, hormonal, or ectohormonal.

Evolution of metazoan chemical coordinating systems. The view that an organism evolves receptor sites in response to some ubiquitous molecules which are adapted to special tasks seems especially applicable to the feeding activators of coelenterates. So long as a molecule was widely present in prey organisms and had properties distinguishing it from closely related substances, it might serve as a feeding activator. One might, therefore, expect some coelenterates to have evolved receptor sites for compounds other than glutathione emitted by the captured prey. Such is the case: some coelenterates have evolved receptor sites for proline (*6, 9*); another has evolved sites for valine (*8*); another, for glutamine (*11*).

After a receptor site for a specific compound had been acquired during evolution, further modification of the receptor site itself might have occurred. For example, Fulton has suggested (*6*) that the evolution of a receptor site for glutathione into one for the α-imino acid proline may have proceeded by means of slight structural changes in the receptor site. He postulated this because one of the possible cyclized forms of glutathione in solution is close in structure to an α-imino acid. And, since proline is also present in the fluids released from prey organisms, the change in structure of the receptor site was not disadvantageous to *Cordylophora* but, under some circumstances, advantageous, and so persisted. Perhaps the coral *Cyphastrea*, which responds to both proline and glutathione (*10*), may represent a form retaining both receptor sites.

I find it reasonable to suppose that receptor sites for glutathione in hydra and for peptide hormones in vertebrates may have evolved in a similar fashion. But the intriguing question is, Did the chemical receptors to environ-

mental compounds in lower forms give rise to the chemical receptors (olfactory, gustatory, or hormonal) of higher forms? Such evolutionary questions are virtually unanswerable. Nevertheless, perhaps there may be an argument in support of the view that they did, at least in the case of peptide receptor sites. In both lower and higher forms there are receptor sites for specific peptides, which, when activated, lead to contraction responses. It would seem simpler for organisms, during evolution, to have modified existing receptor-effector systems to perform new tasks than to have developed a completely new receptor-effector system. One can imagine that internal peptide activators in the more complex organisms would have had to be relatively more complicated, because these organisms could not chance having one of their specialized control mechanisms activated by simple, ubiquitous compounds present in the circulating fluids (*46*).

Such speculations, like most speculations about evolution, cannot be proved, but they may help to make us aware that unifying concepts, tacitly assumed in the case of enzymes and cell organelles, also may apply to the basic aspects of chemical coordinating systems. And, specifically, such speculations emphasize that the behavioral responses of a lower invertebrate to a peptide and some hormonal responses in man to peptides may have many fundamental features in common. By focusing on the primary events of the combination of the activator with the receptor to initiate a series of coordinated responses, we may find new experimental organisms, new approaches, and new insights into universal, yet little understood, chemical control mechanisms.

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47. I thank Drs. J. Clegg, W. Evoy, R. Iverson, L. Kahan, and R. Mariscal for comments on various parts of this article. The research discussed was supported through grants from the National Institutes of Health (GM 11226, GM 12779), the National Science Foundation (GB 5491), and the Nutrition Foundation, and by a career development award (GM 5011).