active protein, measured by ultraviolet absorption, correlates well with the activity curve (Fig. 1). This purified protein seems to be an aggregate of the protein monomer, because application of the protein to the Sephadex G-75 column indicated that the molecular weight of the purified protein, measured by activity and ultraviolet absorption, was more than 80,000 (*8*). Aggregation proceeded during the dialyzing process against acetate buffer of low ionic strength, although this process did not change the activity of the protein.

Purity of the isolated protein was checked with disc electrophoresis on polyacrylamide gel. The protein migrated to the cathode at *p*H 4.5 (Fig. 2) but did not migrate at *p*H 8.3. This electrophoretic behavior was in agreement with that expected from the result obtained with the cation exchange column, and indicated that the protein is basic. A single sharp band accompanied a light diffused band. These bands changed into a single sharp band (Fig. 2) when electrophoresis was carried out in 8*M* urea. This fact indicated that the isolated protein does not contain any protein other than the active component.

An amino-acid analysis of the protein was carried out after the lyophilized protein was hydrolyzed in 6*N* HCl (Table 1). Sugar components comprise 6.7 percent of the protein. The sugar content of each fraction from a carboxymethyl-Sephadex column was determined by the tryptophan-sulfuric acid reaction (Fig. 1, triangles) (*9*). The possibility that the sugars are an impurity was eliminated by the fact that the curve of sugar content correlated well with the chromatographic pattern of the active protein (Fig. 1) and that the sugar content was not changed by dialysis or rechromatography of the fractions on a carboxymethyl-Sephadex column. Paper chromatography of the protein hydrolyzate gave two sugar spots, which were identified as L-arabinose and D-xylose. These results indicated that the active principle of miracle fruit is a glycoprotein.

The sweetening of the subsequent taste of acids was observed at a $5 \times 10^{-8} M$ concentration of the protein solution and reached a maximum at $4 \times 10^{-7} M$. The sweetening effect at high concentration of the protein solution slowly declines over a period up to 2 hours. The purified protein itself has no inherent taste. A mixture of the protein with sour substances initially tastes sour, and slowly changes to sweet if the mixture is held in the mouth for about 1 minute. This fact ruled out the possibility that a complex of the protein with the sour substance itself has a sweet taste. It is believed that the protein binds to receptors of the taste buds and modifies their function. We call the protein "taste-modifying protein."

KENZO KURIHARA
LLOYD M. BEIDLER
Department of Biological Science, Florida State University, Tallahassee

References and Notes

1. W. F. Daniell, *Pharm. J.* **11**, 445 (1852).
2. G. E. Inglett, B. Dowling, J. J. Albrecht, F. A. Hoglan, *J. Agr. Food Chem.* **13**, 284 (1965).
3. Pulp (100 g) from 300 berries was homogenized with 200 ml of distilled water in a Waring blender for 1 minute. The homogenate was centrifuged at 10,000 rev/min for 10 minutes, and the sediment was washed with water once more. The washed sediment was homogenized with 100 ml of 0.1*M* $NaHCO_3$-Na_2CO_3 buffer, *p*H 10.5, for 1 minute. After the homogenate was stirred for 5 minutes, it was centrifuged at 10,000 rev/min. The extraction was repeated three times with the carbonate buffer. All of the preparative experiments in this paper were carried at 0 to 4°C.
4. Combined extracts with carbonate buffer were applied to a column (3.6 by 45 cm) of DEAE-Sephadex A-25 (50 to 140 mesh) buffered with a 0.025*M* phosphate buffer, *p*H 6.0. The column was eluted with 0.1*M* carbonate buffer, *p*H 10.5, and 600 ml of active fraction was collected.
5. The *p*H of the effluent from DEAE-Sephadex column was adjusted to 3.7, and the effluent was placed in dialyzing tube. The powder of Aquacide II (Calbiochem) was placed outside the tube and kept overnight. The volume (600 ml) of the solution in the tube was concentrated to 100 ml.
6. One milliliter of 0.5 percent protein solution (hemoglobin, chymotrypsin, ribonuclease, and cytochrome c) and a solution of taste-modifying protein was applied to a column (1.5 by 61.5 cm) of Sephadex G-75 (0.1*M* phosphate buffer, *p*H 7.0; flow rate 20 ml/hour). Activity was measured with 1 ml of each fraction after dilution with water to 5 ml.
7. P. Andrews, *Biochem. J.* **91**, 222 (1964).
8. An ultracentrifugal pattern of the purified protein showed a single peak, but the pattern diffused quickly.
9. J. Badin, C. Jackson, M. Shubert, *Proc. Soc. Exp. Biol. Med.* **84**, 288 (1953).
10. H. Masamune and Z. Yosizawa, *Tohoku J. Exp. Med.* **59**, 1 (1953).
11. S. M. Partridge, *Nature* **154**, 443 (1949).
12. Supported by NSF grant GB-4068X and AEC grant AT-(40-1)-2690.

24 May 1968; revised 9 August 1968 ■

Sea Urchin Response to Foreign Substances

Abstract. *An assessment of the response of* Strongylocentrotus purpuratus *to substances injected into the coelom shows that (i) foreign molecules are cleared from the coelomic fluid more rapidly than native molecules, (ii) coelomocytes can respond selectively to albumins of human and bovine origin, and (iii) immunization attempts fail to elicit accelerated coelomocyte uptake or accelerated clearance of foreign protein.*

The vertebrate immune response is characterized by (i) increased responsiveness to a foreign substance as a result of prior exposure to it, and (ii) specificity, permitting this response to proceed only against the immunizing substance and closely related substances. No invertebrate has yet been found which possesses both of these two characteristics (*1*). Conceivably, during evolutionary history, one of these characteristics might have developed before the other, and if so, one might expect to find animal species which have in their response to foreign substances only one of these characteristics. Recent evidence suggests that some invertebrates possess a degree of receptor specificity toward antigens (*2*). We report here that the sea urchin (phylum Echinodermata), while not developing a state of increased responsiveness to foreign protein, nonetheless without prior exposure can respond specifically, discriminating between two structurally related proteins. The finding of such discriminative capacity suggests an evolutionary connection between this sea urchin response and the vertebrate immune response, a possibility which seems more plausible because of the known phylogenetic affinities of echinoderms and chordates (*3*).

Sea urchins *Strongylocentrotus pur-*

Table 1. Clearance of radioactive molecules from the coelomic fluid of *S. purpuratus*.

Molecule	No. of animals per group	Protein present (μg/ml, $\pm$ 1 S.D.) at various times after injection			Protein remaining after 22 hours (mean percent)
		1 hour	13 hours	22 hours	
BSA-C^{14}	11	2.30 $\pm$ .24	0.70 $\pm$ .29	0.28 $\pm$ .15	12.2
HSA-C^{14}	6	2.97 $\pm$ .44	0.96 $\pm$ .19	0.31 $\pm$ .10	10.4
S. purpuratus molecules*	4	2.55 $\pm$ .20	1.44 $\pm$ .26	1.36 $\pm$ .19	53.2

* See text for preparation procedure.

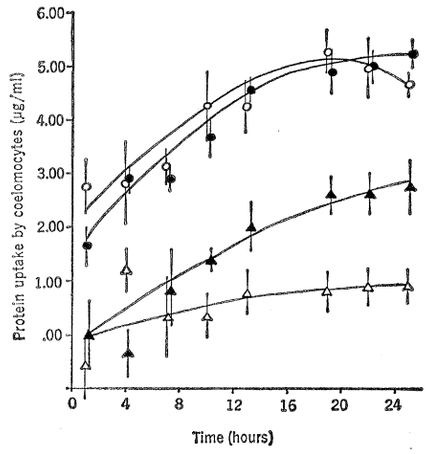

Fig. 1. Comparative uptake of BSA-C^{14} and HSA-C^{14} by coelomocytes of pretreated and control sea urchins. ▲——▲, Uptake of BSA-C^{14}, and △——△, uptake of HSA-C^{14}, by animals pretreated with 100 mg of HSA 2 weeks prior to testing. ●——●, Uptake of BSA-C^{14}, and ○——○, uptake of HSA-C^{14}, by controls. Each group contains six or more animals. Symbols show means, and vertical lines are ±1 standard error. There is a statistically significant difference between the uptakes of BSA-C^{14} and HSA-C^{14} by animals pretreated with HSA at 19, 22, and 25 hours ($P < .01$ in each case).

puratus weighing between 50 and 80 g were used for these experiments. Our approach was to measure the clearance and the uptake by coelomocytes of bovine serum albumin (BSA) and human serum albumin (HSA) labeled with *p*-aminobenzoic acid–C^{14} (PABA-C^{14}) according to the method of Phillips *et al.* (*4*). The specific activities of the proteins varied between 80 and 120 count/min per microgram of protein, depending upon the batch. Test doses of approximately 16 µg of radioactive protein per gram of animal body weight were injected. The clearance of labeled protein which had been injected into the coelomic fluid was determined by removal of samples of 0.5 ml of coelomic fluid through the peristome membrane with a 1-ml syringe fitted with a 26-gauge needle. Each sample was centrifuged at 2000*g* for 20 minutes to remove coelomocytes, and a 0.1-ml aliquot of the supernatant was placed on a ringed aluminum planchet for measurement of radioactivity (*5*). The clearance of BSA-C^{14} closely reflected the clearance of unlabeled BSA, the latter being measured by the method of Lowry *et al.* (*6*). Thus it was clear that the label did not dissociate from the BSA during the course of the experiment.

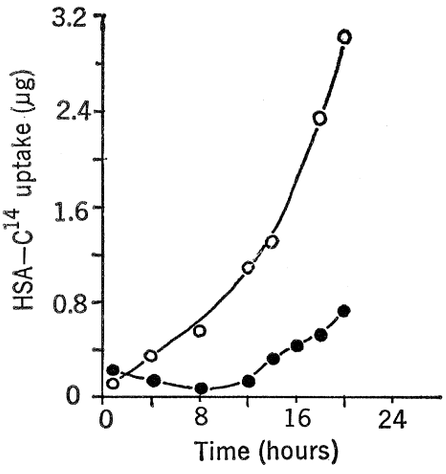

Fig. 2. In vitro uptake of HSA-C^{14} (320 µg/ml) by 5×10^5 coelomocytes in the presence (●——●) or absence (○——○) of added unlabeled HSA (320 µg/ml). Added HSA causes a decrease in uptake of HSA-C^{14}.

The uptake of protein by coelomocytes in vivo was assayed by obtaining the difference in radioactivity between an aliquot of whole coelomic fluid and an aliquot of centrifuged coelomic fluid (cell-free). For the in vitro assays of coelomocyte uptake of protein, cells were maintained in artificial seawater and cell samples were collected on Millipore filter membranes as previously described (*5*). For studies of responses to native molecules, coelomic fluid substances of *S. purpuratus* were labeled by the injection of 20 µc of leucine-C^{14} into the coelomic cavity; the coelomic fluid was then collected 40 hours later. More than 95 percent of the coelomic fluid radioactivity was excluded by Sephadex G-25, indicating that the bulk of the label was in molecules with a molecular weight greater than 25,000.

The clearance of native and foreign molecules is shown in Table 1. While more than 85 percent of the BSA-C^{14} or HSA-C^{14} is removed in 22 hours, less than 50 percent of the *S. purpuratus* molecules are removed. This result suggests that these animals discriminate between native and foreign substances.

Attempts were made to immunize the sea urchins by injecting BSA into the coelom. Injected animals were then tested for their ability to clear BSA-C^{14} from the coelomic fluid and to take up BSA-C^{14} into coelomocytes. Animals receiving 0.01, 0.1, 1.0, 10.0, and 25.0 mg of BSA were tested 2 weeks after injection. Groups of sea urchins receiving 1 mg of BSA were tested at various times after injection: 18 hours, 3 days, 7 days, and 2 months. Finally, a group of urchins received an injection of 1 mg of BSA every week for 6 weeks, and was tested 2 weeks after the last injection. These procedures failed to elicit either accelerated clearance or increased cellular uptake of BSA-C^{14}.

Since immunization attempts failed to provide an altered response which could be analyzed for specificity, attempts were made to alter responsiveness by preinjecting a *large* dose of protein. We have reported (*5*) that sea urchins preinjected with 100 mg of BSA show drastically reduced coelomocyte uptake of BSA-C^{14}. This finding was attributed to the continued presence of BSA from preinjection, saturating receptor sites for BSA-C^{14} but permitting BGG-C^{14} uptake to proceed. The capacity to discriminate between BSA and BGG does not indicate a degree of discrimination comparable to that of the vertebrate immune response. However, a more sensitive test of discriminative capacity was provided by the following experiment involving the two albumins BSA and HSA, which are known to have about the same molecular weight and to share antigenic determinants (*7*). Sea urchins were preinjected with 100 mg of HSA, and 2 weeks later coelomocytes were tested for their ability to take up BSA-C^{14} and HSA-C^{14}. Figure 1 shows that preinjection causes a considerable inhibition of subsequent HSA-C^{14} uptake and a lesser inhibition of BSA-C^{14} uptake. Since preinjected

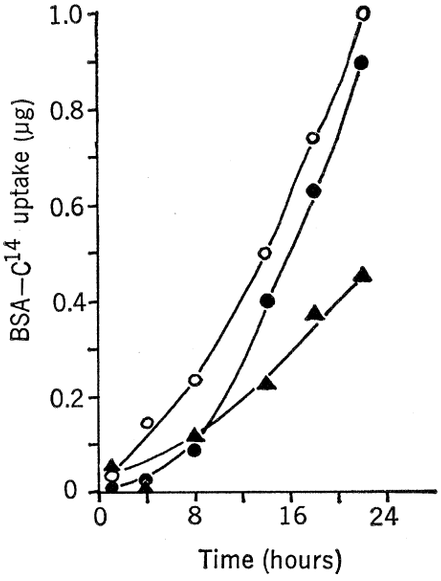

Fig. 3. In vitro uptake of BSA-C^{14} (80 µg/ml) by 5×10^5 coelomocytes: ▲——▲, in the presence of added BSA (80 µg/ml); ●——●, in the presence of added HSA (80 µg/ml); ○——○, without added unlabeled protein. Added BSA, but not added HSA, causes a decrease in uptake of BSA-C^{14}.

animals show HSA still remaining from preinjection (700 ± 50 μg/ml), the inhibition of uptake of labeled HSA can probably be accounted for by isotope dilution. There is no change in coelomic fluid *p*H resulting from preinjection.

This capacity to selectively inhibit uptake was further studied in vitro. In these studies, cells from normal animals were used in the concentration of 5×10^5 per milliliter. Figure 2 shows that HSA (320 μg/ml) added to HSA-C^{14} (320 μg/ml) causes a reduction in the uptake of the HSA-C^{14}. Figure 3 shows that the uptake of BSA-C^{14} (80 μg/ml) is decreased by added BSA (80 μg/ml) but not by added HSA (80 μg/ml). The results of these experiments show that coelomocytes of *S. purpuratus* can discriminate between two closely related proteins.

A model predicting such discrimination has been put forward by Boyden (*8*), and while the results presented here fulfill predictions of his model, they indicate nothing about the nature of the actual uptake mechanism. The constellation of characteristics shown by these experiments is what one might find if one eliminated the proliferating effector cell lines (lymphocytes and plasma cells) from the vertebrate immune response, but retained the effector cell functions in a cell population which exhibits no lasting increase in number of cells or number of receptor molecules per cell as a result of contact with antigen.

HENRY R. HILGARD
Division of Natural Sciences,
University of California,
Santa Cruz 95060

JOHN H. PHILLIPS
Hopkins Marine Station,
Stanford University,
Pacific Grove, California

References and Notes

1. R. A. Good and B. W. Papermaster, *Advan. Immunol.* **4**, 1 (1964).
2. J. H. Phillips, *Ann. N.Y. Acad. Sci.* **90**, 760 (1960); F. B. Bang, *J. Immunol.* **96**, 960 (1966); H. R. Hilgard, W. E. Hinds, J. H. Phillips, *Comp. Biochem. Physiol.* **23**, 815 (1967).
3. A. S. Romer, *The Vertebrate Body* (Saunders, Philadelphia, 1962).
4. J. H. Phillips, S. A. Robrish, C. Bates, *J. Biol. Chem.* **240**, 699 (1965).
5. H. R. Hilgard, W. E. Hinds, J. H. Phillips, *Comp. Biochem. Physiol.* **23**, 815 (1967).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
7. W. O. Weigle, *J. Immunol.* **87**, 599 (1961).
8. S. V. Boyden, in *Cell-Bound Antibodies*, B. Amos and H. Koprowski, Eds. (Wistar Institute Press, Philadelphia, 1963), p. 7.
9. We thank W. E. Hinds for competent assistance. This work was done during the tenure of an established investigatorship of the American Heart Association awarded to H.R.H.

30 July 1968

Calcium Supply to Plant Roots

Abstract. *Direct observations of the calcium-45 distribution around the roots of plants growing in soil showed a pattern in direct contrast to that predicted from calculations based on the calcium concentration of the soil solution, transpiration rates, and plant calcium content. Whereas calcium-45 accumulation at the root surface was predicted, depletion was observed. It is suggested that preferential water movement in larger pores may decrease the expected solution calcium accumulation at the root surface. However, autoradiographs may give no indication of soil solution concentration depletion or accumulation because of the relatively high level on the colloid surface. The nature and extent of the depletion indicates how profoundly the plant root alters its immediate environment in the soil.*

The two processes moving ions from the bulk of the soil to the root are mass flow and diffusion (*1*). These processes are complemented by the effect of plant-root extension. The contribution of mass flow, where ions are passively carried toward the root, has been calculated from the product of the amount of water moving to the root and the concentration of the soil solution. Barber, Walker, and Vasey (*2*) suggested that, on many of the soils they studied, calcium would accumulate around the root as a result of movement of calcium ions by mass flow in the soil solution. This conclusion was based on a range of 1 to 380 parts per million (ppm) calcium in soil solutions extracted from 135 soils of the North Central United States and a figure of 0.3 percent calcium in the corn plant. Al-Abbas and Barber (*3*) found a similar result for soybeans. Their calculations suggested that four times as much calcium reached the root as was absorbed by the plant.

In our experiment, wheat plants, with a calcium level at the end of the experiment (14 days) of 0.30 percent, absorbed an average of 256 μg of calcium per plant, whereas mass flow could have transported 2522 μg of calcium in the soil solution (with a level of 178 ppm in the soil solution, and while the plants transpired at a rate of 170 ml/g). It would appear that these results support the hypothesis of Barber *et al.* in indicating the presence of accumulated calcium at the root surface. But no such accumulation was observed in any of the autoradiographs taken (Fig. 1a). A similar experiment with a lower, but still adequate level of calcium in the soil solution (151 ppm) gave an even more marked depletion zone (see Fig. 1b).

The technique used in this study was similar to that described earlier (*4*). Wheat seeds were sown in soil in a narrow container so that the root grew against a thin mylar film. After the movement of ^{45}Ca in the soil, x-ray film was placed against the mylar overnight. Three wheat seedlings were

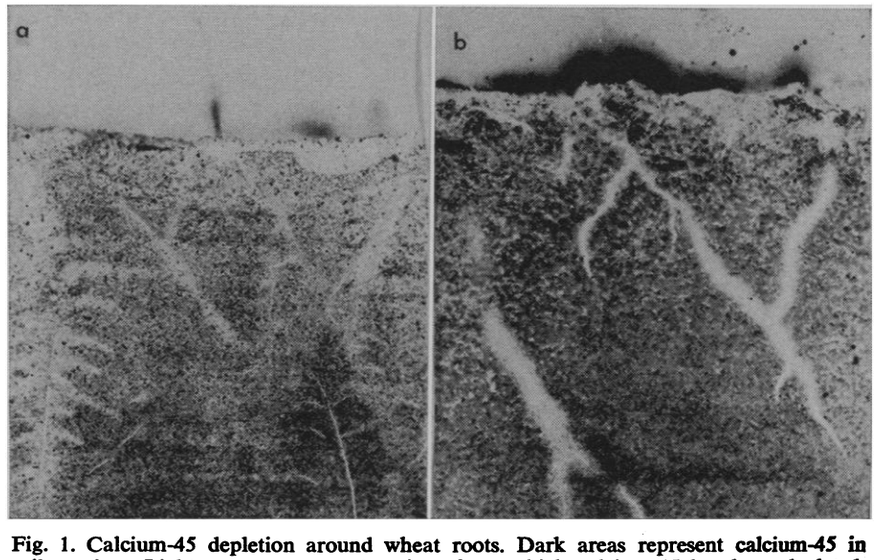

Fig. 1. Calcium-45 depletion around wheat roots. Dark areas represent calcium-45 in soil or plant. Lighter areas represent regions from which calcium-45 has been depleted. (a) In experiment 1, the calcium-ion concentration was 178 ppm in soil solution on day 12. (b) In experiment 2, the calcium-ion concentration was 151 ppm in soil solution on day 8.