STUDIES ON THE RESPIRATORY PIGMENT OF URECHIS EGGS

By N. H. HOROWITZ* and J. PERCY BAUMBERGER

(*From the School of Biological Sciences, Stanford University, California*)

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Experiments previously reported (1) have shown that the eggs of the Pacific marine worm, *Urechis caupo*, contain a reversible oxidation-reduction pigment. The pigment, called *urechrome*, is autoxidizable and changes color from red to yellow on oxidation. It is soluble in water (reduced form insoluble below pH 5) and in acidified methanol, but insoluble in ether, acetone, chloroform, and neutral alcohol. Evidence for participation of the pigment in cellular respiration has been previously given.

Because of its biochemical interest and possible embryological significance, a more extensive study of the substance has been made, the results of which are reported below.

Occurrence and Preparation—Urechrome occurs chiefly in the eggs of the worm. None has been found in the muscle, viscera, egg sacs, or sperm. Traces are found in the centrifuged blood elements of the female, probably in association with the oocytes, which mature in the blood of this species.

To prepare the pigment, the egg sacs are dissected from the animal and dropped into sea water, care being taken to prevent contamination by blood. The eggs are released by puncturing the sacs and the empty sacs are removed. After the eggs have been washed several times, they are centrifuged down and transferred to a Soxhlet apparatus where they are extracted first with acetone and then with ether until yellow pigments no longer come over. The urechrome, which remains behind, is extracted by shaking with 0.1 N HCl-methanol at 40°. The solution is

* National Research Council Fellow in the Natural Sciences, 1939–40. Present address, California Institute of Technology, Pasadena.

filtered and then concentrated *in vacuo* to about one-fourth of the original volume. It is now brought to pH 2 (glass electrode) with 1 N NaOH, precipitating reduced urechrome. After standing at 0° for several hours, the precipitate is centrifuged down, redissolved in HCl-methanol, and reprecipitated with 4 to 5 volumes of ether. The ether precipitation is repeated, after which the pigment is collected in a centrifuge tube, washed twice with absolute methanol, and twice with distilled water. It is dried *in vacuo* over calcium chloride. The reduced pigment dries in dark violet, glistening flakes. It is non-crystalline. 10 gm. of dehydrated, defatted eggs yield about 40 to 50 mg. of urechrome. An additional 15 to 20 mg. can be recovered from the supernatant of the NaOH precipitation by reducing the oxidized pigment, which has remained in solution, with a small crystal of stannous chloride and proceeding as with the first precipitate. It has been found that reduced urechrome can also be precipitated from HCl-methanol with a small amount of pyridine, apparently as the result of salt formation.

EXPERIMENTAL

Oxidation-Reduction Potential—The oxidation-reduction potential has been studied polarographically (2–4), checked by potentiometric titration. The advantage of the polarographic method consists in its being particularly adaptable to small quantities in which the ratios of oxidant to reductant and the corresponding values of E_h are determinable without the addition of a second system. The method as used by us is less accurate than the potentiometric titration, the error being ±5 millivolts.

The instrument used was the Heyrovsky-Nejedly type with a dropping mercury electrode and a saturated calomel reference half-cell. The set-up was essentially that described by Müller and Baumberger (2). The temperature was 25° ± 1°.[1] A saturated solution of pigment (1 to 3 mg. per cc.) in phosphate or borate buffer of the desired pH was deoxygenated by a stream of O_2-free nitrogen. A small quantity of platinized asbestos was added and the gas changed to hydrogen. At various stages of reduction (judged roughly by the color of the solution) the hydro-

[1] Owing to a typographical error, the temperature of the experiments was previously reported as 250° (1).

gen was replaced by nitrogen and a current-voltage curve was photographically recorded (Fig. 1). After complete reduction had been attained, the process could be reversed by introducing successive small amounts of air into the solution until complete oxidation was reached. From the polarographic curves so obtained, E_h and the relative heights of the cathodic and anodic waves can be measured, from which E'_0, the ratio [Ox]:[Red], and the number of electrons involved in the reaction can be calculated.

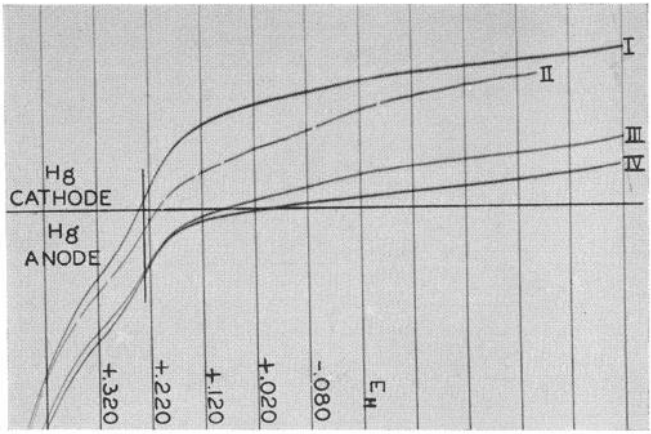

FIG. 1. Polarogram of urechrome. Phosphate buffer, pH 6.26. The horizontal line indicates "galvanometer zero," above which a dropping Hg electrode is the cathode and below which it is the anode. A short vertical line is drawn through the half waves, intersecting "galvanometer zero" at E'_0. The ratios [Ox]:[Red] are as follows: Curve I, 4:3; Curve II, 7:18; Curves III and IV, practically zero.

Details of the calculations are given in the paper of Müller and Baumberger (2). The potentials were readily reproducible, and were not noticeably affected by variations in the total concentration of dye.

The change in E'_0 with pH was investigated from pH 5 to 10. These are the practical limits of pH for the substance, since below pH 5 the reduced form is insoluble and above pH 10 the pigment is unstable. E'_0 decreases by 59 millivolts per unit increase in pH, within the limits of experimental error (Table I).

The reduction of urechrome involves only 1 electron, as can

be seen in Table I, where calculated values of E_h for 1 and 2 electron processes are compared with the observed potentials. With some preparations a second polarographic wave was observed at a potential several hundred millivolts negative to the main wave. The height and slope of this wave were extremely variable, in some cases being pronounced, in others imperceptible. The

TABLE I

Relation of E'_0 to pH and Evaluation of n, from Polarographic Data

pH	E'_0	$\frac{[Ox]}{[Red]}$	E_h		
			Calculated $n = 1$	Calculated $n = 2$	Observed
			volt	*volt*	*volt*
5.31	0.287				
6.26	0.228	4:3	0.236	0.232	0.239
		7:18	0.203	0.215	0.206
7.39	0.163	3:1	0.192	0.178	0.191
		9:7	0.170	0.167	0.172
		5:36	0.112	0.137	0.106
		2:25	0.097	0.130	0.091
		1:23	0.081	0.122	0.077
7.70	0.143*				
8.62	0.089*				
9.15	0.057	7:3	0.079	0.068	0.077
		19:13	0.067	0.062	0.066
		21:16	0.064	0.061	0.063
		2:11	0.013	0.035	0.011
		8:47	0.011	0.034	0.011
		1:23	−0.025	0.016	−0.020
9.97	0.007	7:1	0.058	0.033	0.056
Mean deviation from observed....			0.003	0.019	

* Potentiometric titration.

"half wave" potential did not vary in any regular manner with pH, nor was it ever observed to fall below the "galvanometer zero" line, *i.e.* in the region corresponding to a completely reduced system, even after prolonged bubbling of hydrogen. We believe the wave to be due to an unknown impurity or decomposition product.

As a check on the polarographically determined potentials a few potentiometric titrations with a platinum electrode were carried

out. The reducing agent was $Na_2S_2O_4$, the oxidizing agent ferricyanide. The atmosphere was nitrogen. The results on the whole are in close agreement with the polarographically determined potentials, although the curves tend to be somewhat steeper than the theoretical, with drifting potentials at the extremes of the curve. This is attributed to poor poising of the system. A titration curve at pH 8.62 is shown in Fig. 2. The agreement between the polarographic and titrimetric measure-

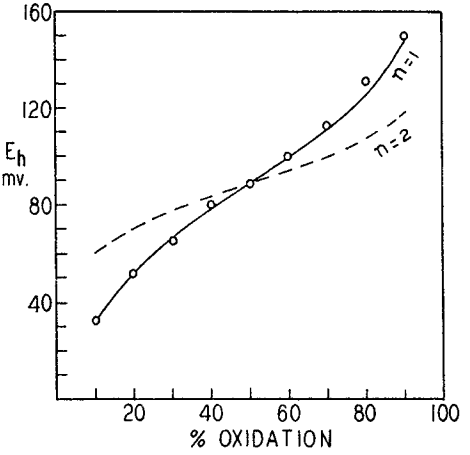

FIG. 2. Titration of reduced urechrome with ferricyanide, pH 8.62. The curves are the theoretical for $n = 1$ and $n = 2$. The circles represent experimental values.

ments is in accord with the findings of previous studies on electroactive reversible systems.

We conclude that urechrome is a reversible oxidation-reduction system conforming, within the experimental limits, to the equation

$$E_h = 0.599 + \frac{RT}{F} \ln \frac{[\text{Ox}]}{[\text{Red}]} + \frac{RT}{F} \ln [\text{H}^+]$$

where the formulation of the reaction is as follows:

$$\text{Oxidant} + \text{H}^+ + e \rightleftharpoons \text{reductant}$$

Absorption Spectrum—Solutions of the pigment exhibit no specific absorption in the visible region. Aqueous solutions of the

oxidized form show general absorption of wave-lengths below 540 mμ, whereas the reduced form absorbs generally below 620 mμ. Reduced urechrome dissolved in acid methanol shows a band in the near ultraviolet at 388 mμ. The oxidized form has not been investigated in this region.

Reduced urechrome forms a deep violet solution in concentrated sulfuric acid. This solution exhibits two marked absorption bands: a broad band centering at 553 mμ and a narrow band at 688 mμ. The bands resemble those of hematoporphyrin anhydride, formed from hemoglobin in concentrated sulfuric acid, which shows a broad band at 553 mμ and a narrow one at 613 mμ. It was therefore attempted to make a pyridine hemochromogen derivative of urechrome by reduction in alkaline solution in the presence of pyridine. The resulting solution showed a strong band at 548 mμ and a weak band at 515 mμ, typical of pyridine hemochromogen. It should be noted that the spectrum of pyridine hemochromogen cannot have resulted from contamination of the preparation with hemoglobin, since no hemoglobin spectrum was observed in untreated solutions, nor is the spectrum obtained by sulfuric acid treatment that of hematoporphyrin.

Molecular Weight—Since the degree of purity of the pigment was not known, the molecular weight was approximated from diffusion measurements. A Northrop and Anson type of diffusion cell (5, 6) of 7.29 cc. capacity was used. The membrane constant, K, determined by standardization against 2 M NaCl, was 0.470. The temperature of the thermostat was 20° ± 0.05°. In order to obtain a high concentration of urechrome in the diffusion cell, acidified methanol was the solvent used in the dye experiments. Relative concentrations on the two sides of the membrane were measured with an Evelyn photoelectric colorimeter.

Three determinations of the diffusion constant of urechrome gave $D_{20,\ \text{methanol}} = 0.363$, 0.366, and 0.377 sq. cm. per day; average, 0.368. Assigning the values 0.0062 to the viscosity of the solvent and 1.3 to the density of the pigment, one arrives at an approximate molecular weight of 1700. The method of calculation is given by Northrop and Anson (5). Since the calculation is based on the assumptions of a spherical molecule and the absence of solvation and ionization effects, the strict validity of which is in most cases doubtful, this value is to be taken as an approximation only.

The molecular weight from diffusion data is in fair agreement with that calculated from hydrogenation experiments. Urechrome is rapidly and reversibly reduced by hydrogen gas in the presence of finely divided platinum. From the potentiometric data it is known that the reduction involves 1 hydrogen atom per molecule of pigment. By carrying out the reaction in Warburg manometers, the uptake of hydrogen can be measured and the equivalent weight of pigment calculated. It was found that 8.18 c.mm. of H_2 are taken up per mg. of pigment. The weight of pigment equivalent to 1 gm. atom of hydrogen is then

$$\frac{\frac{1}{2} \times 22{,}400}{8.18} = 1370$$

DISCUSSION

Urechrome is an autoxidizable pigment of relatively high potential. Evidence for its participation in the respiration of the egg cell has been presented in a previous paper. Because of the difficulty of obtaining sufficient quantities of the substance, further chemical studies of the molecule have not been possible. The available data, however, suggest that it may be related to the heme pigments. Such a hypothesis is consistent with the observed valence change of 1 on reduction, with the absorption spectra of the derivatives studied, and with the solubility properties of the pigment. That it is not a typical heme pigment, however, is evidenced by the absence of characteristic absorption bands in solutions of the untreated dye, as well as by the molecular weight, which appears to be too high for that of a free metalloporphyrin and too low for that of a chromoprotein. The fact that the pigment is not extracted from the cell by aqueous solvents, although it is soluble in them, suggests that, as obtained in extracts, it may represent a fragment of a larger molecule. This would explain the molecular weight and possibly other properties of the substance.

Concerning the biological significance of urechrome, the outstanding fact is that it has been found only in the eggs. Its potential, which is in the range of the cytochromes, and its autoxidizability indicate that it intermediates between oxygen and the reducing systems of the cell. It is noteworthy that spectroscopic examinations made by us of both living eggs and extracts have failed to

reveal the absorption bands of cytochrome. The studies of Brachet (7) and of Ball and Meyerhof (8) on the eggs of other species have given similar results. As is pointed out by the latter authors, this does not constitute proof that the cytochromes are not present, since they may occur in quantities too small for detection by ordinary methods. They suggest the possibility that a hemin other than cytochrome may be involved in the respiration of the egg. The present work constitutes evidence that such may be the case in the eggs of *Urechis*. On the basis of microscopic examinations, Baumberger and Michaelis (9) have concluded that pigmented granules present in these eggs are derived from the hematin of the blood. It appears likely that these granules are to be identified with urechrome, although this would be difficult to prove. It has been observed (10) that during the embryonic development of the egg red granules become localized in the ciliated regions of the embryo, suggesting a relationship with the ciliary metabolism.

SUMMARY

1. Urechrome, the red pigment of the eggs of *Urechis caupo*, is a reversible oxidation-reduction system. At pH 7 $E'_0 = +0.186$ volt; $n = 1$.

2. Solutions of the untreated pigment show no specific absorption in the visible region. In concentrated sulfuric acid bands appear at 553 and 688 mμ. Reduction in alkaline pyridine solution brings out the spectrum of a pyridine hemochromogen.

3. The approximate molecular weight determined from diffusion experiments is 1700.

4. It is suggested that the pigment is related to the hemins.

5. The possible physiological rôle of urechrome is discussed.

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