

CRYSTALLINE ECHINOCHROME AND SPINOCHROME: THEIR FAILURE TO STIMULATE THE RESPIRATION OF EGGS AND OF SPERM OF *STRONGYLOCENTROTUS*

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Communicated August 28, 1939

The blood cells (elaeocytes) of sea-urchins contain a red pigment to which MacMunn¹ gave the name echinochrome. McClendon² found that this pigment is also present in the test and, in the species *Arbacia punctulata*, in the eggs as well. Cannan³ showed that it could be reversibly oxidized and reduced. Vlès and Vellinger⁴ made use of it (called it Arbacin) as a natural pH indicator in the eggs of *Arbacia*. Stern⁵ (referring to unpublished data) considers it to be bound as a chromoprotein in the blood cells.

The present author's interest in echinochrome arose from Friedheim's⁶ report that it would cause a 16-fold increase in the respiratory rate of unfertilized sea-urchin (*Strongylocentrotus lividus*) eggs. In view of the magnitude of the reported effect and the fact that the substance is a naturally occurring pigment, it seemed desirable to examine its action on fertilized sea-urchin eggs. This was of especial interest in connection with studies⁷ on the energetics of development. However, my attempts to duplicate Friedheim's results on unfertilized sea-urchin eggs have proved unsuccessful. Friedheim did not give in his articles the method of preparation except to state that he extracted the tests of *Sphaerechinus granularis* with sea water. In the present work sea water extracts of *Strongylocentrotus purpuratus* were found to be inactive. The material was then prepared in crystalline form, and the purified echinochrome also was found to have no stimulating effect on the respiration of sea-urchin eggs.

Recently another effect has been attributed to echinochrome: Hartmann, Schartau, Kuhn and Wallenfels⁸ have announced that it is the substance causing activation and chemotaxis of the spermatozoa in *Arbacia pustulosa*. They state that the limit of physiological activity lies at a dilution of 1:2,000,000,000. On examination of the action of purified echinochrome on the sperm of *Strongylocentrotus purpuratus* I have found no such action as Hartmann, *et al.*, reported for *Arbacia*.

The crystallization of echinochrome was first reported by Ball.⁹ It was also crystallized by Lederer and Glaser¹⁰ who, though evidently unacquainted with Ball's brief abstract, assigned to it the same empirical formula, C₁₂H₁₀O₇. Lederer and Glaser also obtained another pigment which they call spinochrome and which differs from echinochrome in containing one more atom of oxygen. By a similar procedure I was able to obtain

crystalline spinochrome from *Strongylocentrotus purpuratus*. It, too, showed no stimulating effect on the respiration of the eggs and the spermatozoa.

Preparation of Crystalline Echinochrome and Spinochrome.—Ground sea-urchin tests are first extracted with neutral alcohol and then ether. This removes very little of the echinochrome or spinochrome. The test material is then added in successive small portions to 0.5*N* HCl under ether, fresh portions of concentrated HCl being added as the CO₂ evolution ceases. The pigments go almost quantitatively into the ether layer. Shaking the ether solution with aqueous NaHCO₃ brings the pigments into the hypo-

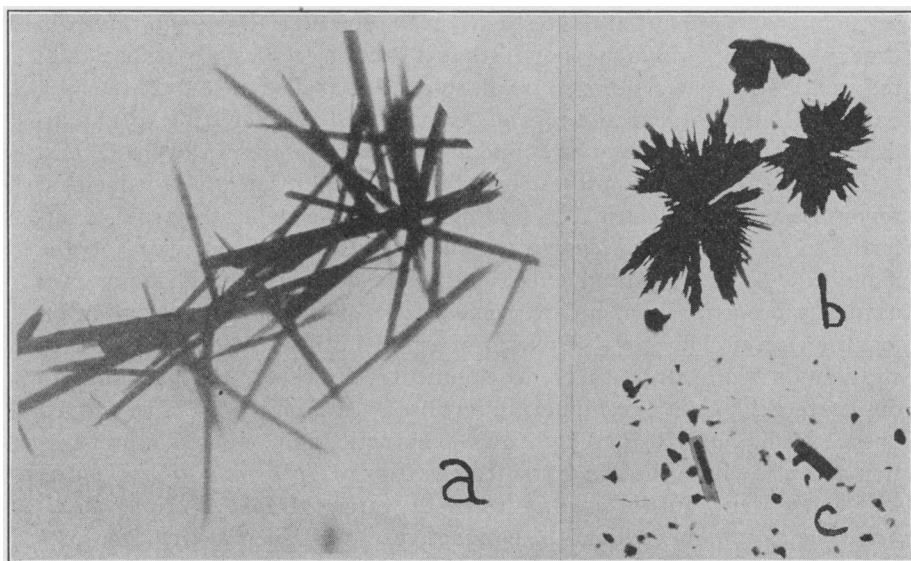


FIGURE 1

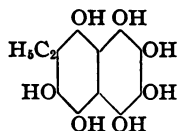
a, Echinochrome crystals $\times 100$; b, Spinochrome crystals in rosettes $\times 50$; c, Spinochrome crystals $\times 100$.

phase, from which they are returned to fresh ether upon acidification. The dried ether solution is then run through a column of CaCO₃. Two main layers appear, an upper bluish violet layer and, below this, a red layer. These two layers remain contiguous even after repeated chromatographic adsorption. The upper layer contains principally the spinochrome, the lower layer the echinochrome. The pigments are "eluted" by dissolving the CaCO₃ in acid under ether. The ether is then dried and evaporated to small volume. The addition of petrol ether precipitates the pigment. The precipitate is then fractionally vacuum-sublimed. The sublimate generally deposits in crystalline form, and the corresponding bands of the chromatogram yield principally echinochrome or spinochrome, respectively.

Recrystallization is effected from hot toluene, hot benzene or evaporation of an ether solution containing ligroin.

The crystals of echinochrome (Fig. 1a) are bright red elongate needles melting at 220° to 221°C. Spinochrome (Fig. 1b, c) forms very thin, pale-violet, elongate plates, strongly birefringent and melting at 187° to 188°C.

The investigations of Ball,⁹ Lederer¹⁰ and Kuhn¹¹ show echinochrome to be a substituted naphthoquinone with the following structure in the leuco form:



In spinochrome the location of the extra atom of oxygen has not as yet been determined.

Both echinochrome and spinochrome are only slightly soluble in water or dilute acid. They go readily into solution in alkali. They are very soluble in alcohol, acetone and ether, less soluble in benzene and toluene, insoluble in petroleum ether. The addition of lime water to an aqueous solution of either of these dyes precipitates them almost completely as the calcium salts. It is evidently in this form that the pigments are present in the test of the sea-urchin. A colorimetric comparison of the initial ether extract with standard ether solutions of the purified material shows the pigment concentrations to be roughly 0.5 mg. per gram of dried test.

Action on Respiration of Unfertilized Sea-Urchin Eggs.—Oxygen consumption was measured with the Barcroft-Warburg apparatus. Conical vessels of 15 cc. total volume and with one side arm were employed. The main chamber contained 3.0 cc. of egg suspension and the side arm 1.0 cc. of the solution to be tested. The egg suspensions and solutions were buffered with 0.01 molar glycylglycine.¹²

To test the action of sea-water extracts, tests of *Strongylocentrotus purpuratus* were ground up in sea water. Upon filtration this gives a pale purple opalescent "solution." The addition of this to unfertilized *Strongylocentrotus* eggs produced no change in their rate of oxygen consumption, as the following figures show:

	A	B
Cu. mm. O ₂ consumed per hour before adding solution...	28.0	29.5
after.....	30.0	29.0

The action of purified echinochrome and spinochrome, prepared as described above, was examined both in ordinary sea water and in calcium-free sea water. In ordinary sea water most of the echinochrome or spinochrome precipitates out, presumably as the calcium salt. The addition of

saturated solutions (plus excess solid) of these substances to unfertilized sea-urchin eggs in sea water produced no marked change in the rate of oxygen consumption, as the following figures show:

	ECHINOCHROME		SPINOCHROME	
Cu. mm./hr. before addition	40.0	37.5	37.5	39.5
Cu. mm./hr. after addition	39.0	38.5	40.0	36.5

In calcium-free sea water at pH 8, fairly concentrated solutions of echinochrome and of spinochrome could be prepared. The action of these solutions was tested over a range of concentrations since there might be an optimum concentration above which no stimulation is obtained, as is the case with the substituted phenols.^{13,14} Echinochrome and spinochrome were added to unfertilized *Strongylocentrotus* eggs in calcium-free sea water to give concentrations ranging from 0.01 mg./cc. to 1.0 mg./cc. To check on the stimulability of the respiratory rate of the eggs in calcium-free sea water the action of pyocyanine was also examined. The following table gives a sample of the data obtained.

	ECHINOCHROME			SPINOCHROME		PYOCYANINE	
Mg./cc.	0.01	0.1	1.0	0.01	0.1	1.0	0.5
Cu. mm./hr. before	36.5	37.5	35.5	36.0	37.0	39.0	38.5
Cu. mm./hr. after	37.0	41.5	40.5	40.5	34.0	35.0	307.5

Although pyocyanine gave an eight-fold rise in the respiratory rate of the eggs in calcium-free sea water, echinochrome and spinochrome failed to show any definite effect.

It is somewhat questionable whether the dyes penetrated the eggs in these solutions. While in the solution, it is difficult to tell if the eggs are stained. Upon removal and washing, the eggs are unstained, which may mean rapid diffusion or failure to penetrate initially. If the solutions are acidified to below pH 3, the eggs are colored red by the dyes, and the color remains upon returning the eggs to ordinary sea water. But the eggs are killed by such treatment.

In the eggs of sea-urchins of the genus *Arbacia*, the echinochrome may very well be present in the form of the chromoprotein, as Stern has indicated is the case in the blood. Possibly the eggs of *Strongylocentrotus* and other forms which do not contain echinochrome also lack the protein capable of combining with it.

Friedheim⁶ reported obtaining stimulation of the respiratory rate of rabbit red blood cells as well as of eggs of *S. lividus*. The present negative results with eggs of *S. purpuratus* are, then, evidently not due to species differences. It would appear that in the crude extracts of *Sphaerechinus* tests employed by Friedheim something other than echinochrome was responsible for the stimulating effect.

Action on the Respiration of Sea-urchin Spermatozoa.—When sea-urchin spermatozoa are placed in calcium free sea water they are rapidly immobil-

ized. For the respiration measurements, the spermatozoa were therefore suspended in ordinary sea water (buffered) and the solutions of the dyes in isotonic NaCl (buffered) were added. As a control, isotonic NaCl alone was added, and, as check on the stimulability of the respiration, an egg water in isotonic NaCl was employed. The addition of the solutions of the dye to sea water results in their precipitation as the calcium salts. With high concentrations of dye practically all of the calcium would be removed from the sea water. With low concentrations, on the other hand, very little dye would remain in solution.

Two sets of experiments were run, one with low and one with high concentrations of the dye. With low concentrations (10 γ /cc.) of echinochrome and spinochrome, there was no noticeable effect on the rate of oxygen uptake of the spermatozoa. With high concentrations of the dyes (10 mg./cc.), the rate is definitely inhibited as the data in the following table show.

		ECHINOCHROME		SPINOCHROME		EGG WATER		ISOTONIC NACL (CONTROL)
Cu. mm. O ₂ before addition	0 to 1/2 hr.	103	102	97	98	98	98	99
	1/2 to 1 hr.	81	83	73	74	77	77	77
	1 to 1 1/2 hr.	59	69	59	57	56	57	57
Cu. mm. O ₂ after addition	1 1/2 to 2 hr.	8	11	12	12	57	41	41
	2 to 2 1/2 hr.	7	8	3	7	37	35	35
	2 1/2 to 3 hr.	13	11	1	2	26	21	21
	3 to 3 1/2 hr.	12	11	4	3	22	19	19

The activity of spermatozoa decreases with age of the suspensions, as has long been known. Along with that, the respiratory rate falls off as the above figures (control column and first three readings of the others) show. The addition of egg water causes an increase of about 40 per cent in the respiratory rate for the first half hour and practically no increase for the subsequent readings. The concentration of egg water employed was such as to give an agglutination reaction lasting 15 minutes. The sharp decrease in respiratory rate following the addition of echinochrome and spinochrome is evidently due to the removal of calcium. Most of the dye was observed to precipitate out upon addition to the sea water sperm suspension in the vessels. This precipitate is very likely the calcium salt, since as mentioned before echinochrome and spinochrome form insoluble salts with calcium. With magnesium, which is present in higher concentrations than calcium in sea water, no insoluble salt forms. The 3.0 cc. of sea water contains roughly 3×10^{-5} moles of Ca^{++} . In the 1.0 cc. of echinochrome solution added there are 3.8×10^{-5} moles of the dye, and in the spinochrome solution, 3.5×10^{-5} moles. Since one mole of echinochrome or spinochrome might combine with $2\frac{1}{2}$ moles of calcium, most of the calcium would be removed from the solution. Examination of the

suspensions after the respiration measurements showed the spermatozoa to be almost completely inactive in the vessels with the dyes. This inactivation occurs in 2 to 3 minutes in concentrations of the dyes employed here.

The difference between the present results and those reported by Hartmann, *et al.*,⁸ may possibly be due to the fact that *Arbacia* eggs contain echinochrome whereas *Strongylocentrotus* eggs do not. It should be noted, however, that *Strongylocentrotus* egg water increases the activity of spermatozoa of the same species while echinochrome and spinochrome show no such action. The activating agent in the egg water of this sea-urchin as well as in the keyhole limpet is rather intimately associated with the sperm agglutinin which recent evidence¹⁵ shows to be protein. Partial purification of the latter has not as yet removed the activating agent.

¹ MacMunn, C. A. *Quart. Jour. Micr. Sci.*, **25**, 469 (1885).

² McClendon, J. F., *Jour. Biol. Chem.*, **11**, 435 (1912).

³ Cannan, R. K., *Biochem. J.*, **21**, 184 (1927).

⁴ Viès, F., and Vellinger, E., *Bull. Inst. Océanogr.*, No. 513 (1928).

⁵ Stern, K. G., *Cold Spring Harbor Symp. Quant. Biol.*, **6**, 286 (1938).

⁶ Friedheim, E. A. H., *Arch. Sci. Phys. et Nat. (Geneva)* **14**, 179 (1932); *C. R. Soc. Biol.*, **111**, 505 (1932).

⁷ Tyler, A., *The Energetics of Embryonic Differentiation*, Hermann et Cie., Paris (1939).

⁸ Hartmann, M., Schartau, O., Kuhn, R., and Wallenfels, K., *Naturwissenschaften*, **27**, 433 (1939).

⁹ Ball, E., *Jour. Biol. Chem.*, **114**, p. VI (1936).

¹⁰ Lederer, E., and Glaser, R., *C. R. Acad. Sci.*, **207**, 454 (1938).

¹¹ Kuhn, R., and Wallenfels, K., *Ber. des deutsch. Chem. Ges.*, **72**, 1409 (1939).

¹² Tyler, A., and Horowitz, N. H., *Science*, **86**, 85 (1937).

¹³ Clowes, G. H. A., and Krahl, M. E., *Jour. Gen. Physiol.*, **20**, 145 (1936).

¹⁴ Tyler, A., and Horowitz, N. H., *Biol. Bull.* **75**, 209 (1938).

¹⁵ Tyler, A., and Fox, S. W., *Science* (in press).

THE FINITE STIELTJES MOMENTUM PROBLEM

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Communicated July 24, 1937

I. *Introduction.*—In view of the frequent application of Ritz's method to the solution of eigenvalue problems $L\Psi = \lambda\Psi$ increasing attention has been paid to the study of types of function sets which facilitate the performance of variational calculations. In several numerical calculations it was noted^{1, 2, 3} that the iterated function set $L^i\psi^4$ lead to relatively simple matrix elements of the form $L_n = (\psi, L^n\psi)$ and that the convergence of the