STUDIES ON SHELL FORMATION. VI. THE EFFECTS OF DINITROPHENOL ON MANTLE RESPIRATION AND SHELL DEPOSITION [1, 2]

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Shell formation in mollusks concerns the elaboration of an organic framework or matrix and the orderly growth of calcium carbonate crystals within this matrix. This matrix is composed of three fractions: a water-soluble protein, a scleroprotein, and a polypeptide (Grégoire, Duchâteau and Florkin, 1955). Synthesis and secretion of matrix and any active transport of inorganic ions involved in shell deposition will require metabolic energy. Glycolysis (Humphrey, 1950) and the utilization of tricarboxylic acid cycle substrates (Humphrey, 1947; Humphrey and Jeffrey, 1954; Cleland, 1951; Jodrey and Wilbur, 1955) have been demonstrated in oyster tissues and would lead to the formation of high energy phosphate compounds. If these high energy phosphate compounds are utilized in shell deposition, then dinitrophenol (DNP) by preventing phosphorylation (Hunter, 1951; Lardy and Wellman, 1953; Shacter, 1955) or reducing the tissue concentration of the compounds already formed (Hunter, 1951; Shacter, 1955) may be expected to retard shell deposition. This problem has been examined in the oyster *Crassostrea virginica*. The action of dinitrophenol was studied with respect to (1) respiration of shell-forming tissue; (2) calcium deposition using radioactive calcium as an indicator; and (3) shell regeneration in the whole oyster.

Methods

Measurements of the effect of DNP on respiration were carried out by the Warburg method using a strip of tissue about one cm. wide taken from the posterior portion of the mantle. Endogenous respiration was measured in sea water for 30–60 minutes. DNP was then added from the sidearm, and measurements were continued for periods of one to six hours. DNP solutions were buffered at pH 8 with 0.03 *M* glycine.

The isolated mantle-shell preparation (Hirata, 1953) was utilized for measurement of Ca^{45} deposition as described by Jodrey (1953). These mantle-shell preparations were placed in one liter of sea water containing DNP for one hour. Ca^{45} (one ml. of high specific activity) was then added and the preparations remained in this solution for an additional six hours. Reversibility of DNP action on calcium deposition was tested by first immersing mantle-shell preparations in DNP solu-

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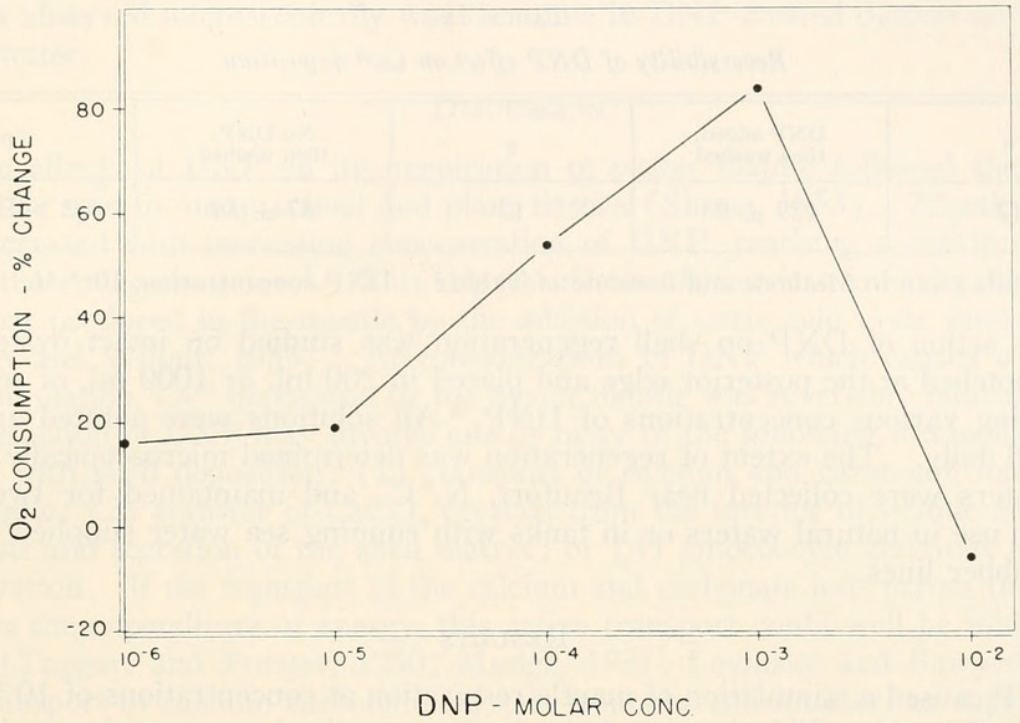

FIGURE 1. Effect of DNP on mantle respiration. Tissue weight, 180–220 mg. wet weight; temperature, 25.6° C.; pH 8.0; DNP, 0.2 ml. in sidearm; total volume 2.2 ml.; gas phase, air; salinity, 33.6–35.4 ‰; mean oxygen consumption of control, 15.6 μl O_2 per 100 mg. wet weight per hour.

tions for seven hours and then washing them in running sea water for two hours. The preparations were then placed in sea water containing Ca^{45} for six hours and the deposition of Ca^{45} was measured. Control preparations were carried through the same procedures but without DNP.

TABLE I

DNP effect on Ca^{45} deposition

DNP conc.	*n*	DNP added counts/min.*	*n*	No DNP counts/min.	P
10^{-3} *M*	5	35 ± 24	5	323 ± 179	<0.01
10^{-4} *M*	12	69 ± 37	12	193 ± 143	<0.01
10^{-5} *M*	9	376 ± 171	9	481 ± 205	0.3 > P > 0.2

Figures in columns three and five show mean calcium deposition by isolated mantle-shell preparations with standard deviations; *n* gives the number of cases. The concentration of Ca^{45} added per liter varied from 5.25 μc to 12.75 μc. For uniformity of presentation calcium deposition is expressed as counts per minute per 10 μc added per liter sea water per 6.2 cm.^{2} of shell. Temperature 25–26° C.; salinity 31.7–34.0 ‰. The experiments were not all carried out at the same time which probably accounts for the differences between the three mean values in column five.

* Shells without mantle were included along with the mantle-shell preparations in these experiments in order to obtain a measure of Ca^{45} exchange. However, it is not certain that the concentration of calcium for exchange is the same for both (Wilbur and Jodrey, 1952), and accordingly exchange corrections have not been made in the figures in Table I. When exchange values are subtracted from the deposition in the mantle-shell preparations the statistical significance is essentially that given.

TABLE II

Reversibility of DNP effect on Ca^{45} deposition

n	DNP added, then washed	*n*	No DNP, then washed	P
12	129 ± 93	12	87 ± 34	$0.2 > P > 0.1$

Details given in Methods and footnote of Table I. DNP concentration, 10^{-4} *M*.

The action of DNP on shell regeneration was studied on intact oysters with shells notched at the posterior edge and placed in 200 ml. or 1000 ml. of sea water containing various concentrations of DNP. All solutions were aerated and were changed daily. The extent of regeneration was determined microscopically.

Oysters were collected near Beaufort, N. C., and maintained for two weeks prior to use in natural waters or in tanks with running sea water supplied through hard rubber lines.

RESULTS

DNP caused a stimulation of mantle respiration at concentrations of 10^{-3} *M* and 10^{-4} *M* (Fig. 1). This increased respiration remained constant for at least six hours. The concentration of DNP which caused maximum respiratory stimulation (10^{-3} *M*, Fig. 1) gave essentially complete inhibition of Ca^{45} deposition by mantle-shell preparations (Table I). The same relationship of respiratory stimulation and inhibition of Ca^{45} deposition held true for 10^{-4} *M* DNP (Fig. 1 and Table I). In further experiments the inhibitory action of 10^{-4} *M* DNP on Ca^{45} deposition was found to be reversible (Table II). When the concentration of DNP was lowered to 10^{-5} *M* and 10^{-6} *M*, there was no statistically significant effect on mantle respiration (Fig. 1). Likewise, at 10^{-5} *M*, DNP had no significant effect on the deposition of Ca^{45} by the mantle-shell preparations (Table I).

Shell regeneration in the presence of DNP was inhibited as the concentration of the inhibitor was increased (Table III). The inhibitory concentrations of DNP were about the same for both calcium deposition by the mantle-shell preparation and for regeneration in the whole oyster (*cf.* Tables I and III). The last column in Table III indicates that DNP is toxic. However, of 20 oysters which were dead by the eleventh day of DNP treatment (all DNP concentrations), 10 showed regeneration. In those cases in which regeneration occurred, the structure of the

TABLE III

Effect of DNP on shell regeneration

DNP conc.	*n*	Oysters showing regeneration in DNP	Comments
0	10	10	No deaths in 14 days
10^{-5} *M*	10	8	1 dead after 14 days
5×10^{-5} *M*	10	7	9 dead after 11 days
10^{-4} *M*	10	3	9 dead after 5 days

Temperature 24.0–29.5° C.; pH 7.7–8.1; salinity 18.0–35.99 ‰. Regeneration occurred in both valves in all but one case.

shell as observed microscopically was the same in DNP-treated oysters and oysters in sea water.

Discussion

The effects of DNP on the respiration of oyster mantle followed the general pattern as seen in many animal and plant tissues (Simon, 1953). Mantle respiration increased with increasing concentration of DNP, reaching a maximum 87% above the endogenous level. This degree of stimulation was considerably higher than that produced in the mantle by the addition of citric acid cycle intermediates (Jodrey and Wilbur, 1955). At concentrations of DNP which caused a respiratory stimulation, Ca^{45} deposition by the oyster mantle was reversibly inhibited.

The action of DNP may involve one or more of the following mechanisms concerned with shell deposition: (1) transport of calcium and carbonate ions across the mantle; (2) transfer of $CaCO_3$ crystals from the interior of mantle cells; (3) synthesis and secretion of the shell matrix; or (4) amoebocyte activities and shell regeneration. If the transport of the calcium and carbonate ions across the mantle requires the expenditure of energy, this active transport could well be inhibited by DNP (Taggart and Forster, 1950; Mudge, 1951; Levinsky and Sawyer, 1953). The transport of calcium carbonate crystals from the cell interior, as suggested by Bevelander (1953), might utilize an energy mechanism susceptible to the action of DNP. In the regeneration experiments failure of intact oysters to deposit matrix alone in the presence of DNP may be due to an effect on matrix synthesis since peptide bond and protein synthesis are known to be inhibited by DNP (Borsook, 1954; Tarver, 1954; Siekevitz, 1952). However, in view of the toxicity of DNP (Table III) one should not conclude that failure of regeneration reflects only a direct effect on matrix synthesis or secretion.

Another possibility arises from the fact that in the snail *Helix aspersa*, amoebocytes play a part in shell regeneration and are thought to be responsible for the deposition of matrix and calcium carbonate crystals (Wagge, 1955). However, the role of amoebocytes in oyster shell regeneration has not been studied and accordingly we do not know their significance with respect to the inhibition of regeneration by DNP.

Summary

1. Dinitrophenol stimulated oyster mantle respiration at 10^{-3} *M* and 10^{-4} *M*. Respiration was not significantly different from the endogenous rate at 10^{-2} *M*, 10^{-5} *M* and 10^{-6} *M* DNP.

2. Calcium deposition, as measured by Ca^{45}, was inhibited in isolated mantle-shell preparations at 10^{-3} *M* and 10^{-4} *M* DNP, the same DNP concentrations which stimulated respiration. Inhibition was found to be reversible.

3. Shell regeneration in whole oysters was inhibited by DNP. DNP concentrations which inhibited regeneration were toxic.

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