

## ACID PRODUCTION AFTER FERTILIZATION OF SEA-URCHIN EGGS

A RE-EXAMINATION OF THE LACTIC ACID HYPOTHESIS

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### INTRODUCTION

Sea-urchin eggs produce acid, the fertilization acid, for a short time after fertilization. Runnström (1933) was the first to describe this phenomenon; he found an acid production equivalent to about 150  $\mu$ l. CO<sub>2</sub>/ml. eggs for the first 20 min. after fertilization in *Paracentrotus lividus*. According to Laser & Rothschild (1939), the r.q. of sea-urchin eggs (*Psammechinus miliaris*) is about 0.65 immediately after fertilization and, using this value, they observed an acid production equivalent to 150  $\mu$ l. CO<sub>2</sub>/ml. eggs during the first 10 min. after fertilization. This figure is based on the curves in fig. 6 of Laser & Rothschild's paper, though reference to the rate curves in the same paper and Rothschild's experiments (1956, fig. 9) shows that even higher rates of acid production may occur during the first 5 min. after fertilization.

Runnström's results and those of Laser & Rothschild were obtained by manometric methods. The positive pressures observed after fertilization of sea-urchin eggs in the absence of KOH were assumed to be due to the evolution of CO<sub>2</sub> following the diffusion of acid out of the eggs and its reaction with bicarbonate in the surrounding sea water. For a number of reasons, including the reported pH, 6.6, of echinoderm egg cytoplasm (Needham & Needham, 1926; Chambers & Pollack, 1927), the evolution of excess CO<sub>2</sub> after fertilization is unlikely to be caused by the production of an acid within the egg and its reaction with bicarbonate within the egg. This question can, however, be settled by experiments, which are described in this paper; they show that the sea water surrounding fertilized sea-urchin eggs has a markedly reduced bicarbonate content.

Runnström (1933) said that the fertilization acid was not lactic acid. Örström & Lindberg (1940), using unpublished results of Runnström, reported values of 0.97  $\pm$  0.07 mg. lactic acid/100 mg. unfertilized egg N and 0.99  $\pm$  0.1 mg. lactic acid/100 mg. fertilized egg N (5-12 min. after fertilization), which quantitatively confirmed Runnström's observations. Yčas (1950) also failed to find any increase in lactic acid production after fertilization. Some years ago I came to the same conclusion and, like other workers presumably, failed to identify the fertilization acid in spite of intensive investigations. The possibility of it simply being H<sub>3</sub>O<sup>+</sup> has been discussed elsewhere (Rothschild, 1956, pp. 65-6).

In the light of these observations, a recent paper by Aketa (1957), in which the fertilization acid is claimed to be lactic acid in the eggs of the Japanese sea-urchin, *Hemicentrotus pulcherrimus*, is of special interest. Aketa obtained a maximum of 120  $\mu\text{g}$ . lactic acid\*/mg. protein N in the first 2 min. after fertilization. Aketa stated that the period of maximum lactic acid production occurred 2–5 min. after insemination and that after this the production rapidly fell. He said that the most probable reason why previous workers had failed to identify the fertilization acid was because they had not made their lactic acid estimations soon enough after fertilization. Later, e.g. 10–20 min. after fertilization, the lactic acid is apparently oxidized. This argument is difficult to reconcile with the fact that acid production, as evidenced by the evolution of  $\text{CO}_2$  from sea water, continued for 20 min. in Runnström's experiments (1933), for at least 5 min. in those of Laser & Rothschild (1939) and for 15 min. in the experiment illustrated by Rothschild in *Fertilization* (1956). These observations are made with the realization that at the low manometric shaking rates imposed by the fragility of sea-urchin eggs there may be some delay in the evolution of  $\text{CO}_2$  from sea water.

Aketa's results raise further difficulties. The unfertilized eggs were inseminated at  $t=0$ ; they were then centrifuged and the supernatant sea water was removed; at  $t=2$  min., trichloroacetic acid (TCA) was added. As the supernatant sea water was discarded, Aketa must mainly have measured lactic acid *inside* the sea-urchin eggs and not in the surrounding medium. If this is so and, if it is true that the fertilization acid diffuses out of the eggs into the surrounding sea water, the lactate found by Aketa within the eggs was, in all probability, not the fertilization acid. The same conclusion can be reached in another way. Suppose that the fertilization acid is lactic acid and that it diffuses out of the egg at fertilization. Why should it disappear or be oxidized in sea water? Alternatively, if there is so much lactic acid within the eggs, why should the lactic acid in the sea water diffuse back into the eggs and be oxidized? These considerations make it unlikely that the fertilization acid is lactic acid, as Aketa believes; but his experiments emphasized the desirability of making a re-examination of this question, which is the subject of this paper.

#### MATERIAL AND METHODS

The eggs of *Echinus esculentus* were used, the jelly being removed by treatment with acidified sea water according to the method recommended by Hagström (1956). A concentrated egg suspension was prepared containing about  $10^5$  eggs/ml. sea water, i.e. 0.2 ml. eggs/ml., the radius of these eggs being *c.* 80  $\mu$ .† After testing the eggs for concentric fertilization membrane formation, 3 ml. of the suspension was

\* Misprinted on p. 269 as 'mg. lactic acid'. In a letter dated 11 June 1958, Dr Aketa kindly gave me some information which indicates that 120  $\mu\text{g}$ . lactic acid/mg. protein N is equivalent to about 1.4 mg. lactic acid/ $10^8$  eggs, or 10 mg. lactic acid/100 mg. N, which is ten times Runnström's value, cited by Örström & Lindberg (1940).

† Dr H. Barnes kindly estimated the total N content of these eggs for me and obtained the value 67 mg. N/ $10^8$  eggs. Ballentine (1940) found 5.8 mg. N/ $10^8$  eggs of *Arbacia punctulata*, whose radius is 37  $\mu$  (Harvey, 1956). The ratio of these values, per ml. of eggs, is 1.01.

put into a conical Barcroft Warburg manometer vessel, vol. c. 20 ml., with 0.33 ml. of sperm suspension in the side-arm. There was no KOH in the centre cup. After the usual equilibration period at 16° C., the spermatozoa were mixed with the eggs, final sperm density  $2 \times 10^7$ /ml., and readings were taken at 2, 5, 10 and 15 min. The experiment was discontinued unless significant acid production, as evidenced by a pronounced positive pressure, occurred. Assuming an R.Q. of 0.7 after fertilization, and that the ratio

$$\{CO_2 \text{ (acid production)} + CO_2 \text{ (respiration)}\} / (O_2)$$

is 1.3 (Laser & Rothschild, 1939), it can be shown (see Appendix) that with the constants applicable to a manometer vessel of standard size containing 3.33 ml. fluid ( $k_{CO_2} = 1.6$ ,  $k_{O_2} = 1.4$ ) any positive pressure observed will be about one-third of the amount, in  $\mu$ l., of the non-respiratory  $CO_2$  evolved.

If the manometric pilot experiment was successful, in the sense that a pronounced positive pressure occurred after fertilization, 1 ml. of the parent egg suspension, which had been aerated meanwhile, was removed for an egg count. 10 ml. of egg suspension were then put into each of four 50 ml. beakers, called 1, 2, 3 and 4. Two of these were fertilized and treated with TCA 2 and 5 min. after insemination. The other two, the unfertilized egg controls, were also treated with TCA 2 and 5 min. after the beginning of the experiment. Table 1 shows how the experiment was done. The times after insemination at which TCA was added were varied according to the rate of acid production observed in the pilot experiments, again bearing in mind that the evolution of  $CO_2$  from sea water is likely to lag behind acid production because of the low shaking rate, 90 c.p.m., which is necessary.

Table 1. *Procedure for examining the lactic acid production of fertilized and unfertilized eggs of Echinus esculentus. T° C., 16*

Egg suspension	1	2	3	4
Time (min.)				
0	+ Spermatozoa	+ Spermatozoa	—	—
2	+ TCA	—	+ TCA	—
5	—	+ TCA	—	+ TCA
6	—	—	+ Spermatozoa	+ Spermatozoa

Final concentrations,  $2 \times 10^7$  sperm/ml. and 5.5% TCA in each case.

In the experiment described above one ends up with: (i) fertilized eggs + spermatozoa fixed 2 min. after insemination; (ii) unfertilized eggs, fixed at the same time as (i), + spermatozoa; (iii) fertilized eggs + spermatozoa, fixed 5 min. after insemination; (iv) unfertilized eggs fixed at the same time as (iii), + spermatozoa. This material was homogenized in a tissue homogenizer (Umbreit, Burris & Stauffer, 1957, p. 171) after which it was centrifuged; the lactic acid content of the supernatant was estimated by the method of Barker & Summerson (1941). Standards containing the appropriate amounts of zinc lactate in sea water were freshly pre-

pared for each experiment and treated in the same way as the experimental material except that, for obvious reasons, homogenization was not necessary.

The mixing operations described above were done in the same way as those involving known sperm-egg interaction times (Rothschild & Swann, 1952; Rothschild, 1953). Both the fertilized and unfertilized eggs require continual agitation to ensure oxygenation.

### RESULTS

*Diffusion of acid out of fertilized eggs into medium.* As mentioned in the Introduction, the conditions in which Aketa did his experiments were such that the lactic acid he measured after fertilization of the eggs had not diffused into the surrounding sea water. This raised the remote possibility that the fertilization acid does not diffuse out of the eggs and that the excess  $\text{CO}_2$  evolved after fertilization comes from bicarbonate within the eggs and not from the sea water round them. To test this possibility, a suspension of jelly-free eggs was divided into two parts, each containing the same number of eggs in 10 ml. of suspension. At  $t=0$ , one suspension was added to 1 ml. of sperm suspension and the other to 1 ml. of sea water. The suspensions were agitated to the same extent as in a manometric experiment and, after 6 min., were transferred to centrifuge tubes and centrifuged for 1 min. in a hand centrifuge. 3 ml. of supernatant was removed from each tube and placed in the main compartments of manometer flasks whose side-arms contained 0.4 ml. 0.1N-HCl. After temperature equilibration, the acid was tipped in each case into the sea water and the manometers were violently shaken for 25 min. The results of two experiments are shown in Table 2. They show without doubt that after fertilization something, which must be an acid, diffuses out of these sea-urchin eggs and reacts with the bicarbonate in the sea water with evolution of  $\text{CO}_2$ . If sea water has a salinity of 32.52‰, the molality of bicarbonate in it is  $2.285 \times 10^{-3}$  (Barnes, 1954), so that the theoretical  $\text{CO}_2$  yield from 3 ml. is 152  $\mu\text{l}$ . Violent aeration for many hours is needed to remove all the bicarbonate- $\text{CO}_2$  from acidified sea water, which is the main explanation of the difference between 152  $\mu\text{l}$ . and the values in the second column of Table 2.

Table 2. *Bicarbonate content, expressed in  $\mu\text{l}$ .  $\text{CO}_2$ , of 3 ml. of sea water round unfertilized (U) and fertilized (F) eggs of Echinus esculentus, seven minutes after insemination.  $T^\circ\text{C}$ ., 16*

Exp.	U	F
1	129	84
2	138	96

*Lactic acid production.* The results of all experiments are given in Table 3, from which the following conclusions may be drawn:

(1) The difference between the amount of lactic acid produced by fertilized and unfertilized eggs is negligible.

(2) The amount of lactic acid produced by fertilized eggs during the first few minutes after fertilization is inadequate to account for the total acid produced during this time. Very roughly, lactic acid production would have to be increased by a factor of 5 to justify the contention that it is the fertilization acid. The discrepancy is all the more marked because the eggs of this sea-urchin produce less fertilization acid than do those of *Psammechinus miliaris*.

Table 3. *Lactic acid and other acid production by fertilized (F) and unfertilized (U) eggs of Echinus esculentus. T° C., 16*

(1) Exp. no.	(2) Time after fertilization (min.)	(3) µg. lactic acid/ml. eggs		(4) Column (3) F, as µl. CO <sub>2</sub> /3 ml. egg suspension.	(5) µl. CO <sub>2</sub> (acid) evolved/3 ml. egg suspension*
		F	U		
1	2	29	34	5	—
2	2	14	15	3	15 (5 min.)
3	5	16	14	2	23
4	5	19	19	3	16
	10	23	22	4	15
5	5	10	9	2	32
	10	8	6	2	17
6	2	16	16	5	1
	5	16	16	5	16
7	2	9	9	2	3
	5	15	15	3	25
8	5	19	12	3	15
	10	18	14	3	27

\* See p. 845 and Appendix for the derivation of the figures in this column.

It is difficult to conceive of any reason why the experimental procedure described in this paper could be responsible for the difference between Aketa's results and those described above; one experiment was, however, done in exactly the same way as were Aketa's, described in the Introduction to this paper. In this experiment the lactic acid found in unfertilized eggs was 12 µg./ml. of eggs and in fertilized eggs 9 µg./ml. of eggs. The average values for unfertilized and fertilized eggs (Table 3) were 15 and 16 µg./ml. of eggs. These results provide some support for the suggestion, made on p. 844, that such lactic acid as is formed in sea-urchin eggs does not diffuse out of them into the surrounding sea water. (The so-called retention of lactic acid is discussed by Umbreit *et al.* (1957) in *Manometric Techniques*.)

#### DISCUSSION

The experiments described in this paper show that there is a marked difference between the eggs of *Echinus esculentus* and *Hemicentrotus pulcherrimus* in regard to (a) the quantity of lactic acid produced by fertilized eggs, (b) the relative quantities of lactic acid produced by fertilized and unfertilized eggs (in *Echinus esculentus* the quantities are the same) and (c) the discrepancy between the observed production

of lactic acid and of fertilization acid. In the light of these findings, together with those of Runnström (1933), Örstöm & Lindberg (1940) and of Yčas (1950), the conclusion is inescapable that the fertilization acid is not lactic acid, even if this substance is produced in unusually large quantities by the fertilized eggs of *Hemicentrotus pulcherrimus*.

## SUMMARY

1. When sea-urchin eggs are fertilized an acid, the fertilization acid, diffuses out of them into the surrounding sea water. A claim has recently been made that the fertilization acid may be lactic acid, which is oxidized shortly after its production.
2. There is no significant difference between the amounts of lactic acid produced by unfertilized and just-fertilized eggs of *Echinus esculentus*.
3. The amount of lactic acid produced by fertilized eggs of this species is too small to explain the evolution of CO<sub>2</sub> observed when the eggs are fertilized in manometer vessels.
4. The sea water round just-fertilized eggs was found to contain markedly less bicarbonate than the sea water round unfertilized eggs, confirming that an acid diffuses out of the eggs after fertilization.
5. There is some evidence that the small quantities of lactic acid produced by unfertilized and just-fertilized eggs of this species do not diffuse into the surrounding sea water.
6. It is concluded that the fertilization acid is not lactic acid.

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## REFERENCES

- AKETA, K. (1957). Quantitative analyses of lactic acid and related compounds in sea urchin eggs at the time of fertilization. *Embryologia*, **3**, 267-478.
- BALLENTINE, R. (1940). Total nitrogen content of the *Arbacia* egg. *J. Cell. Comp. Physiol.* **15**, 121-2.
- BARKER, S. B. & SUMMERSON, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.* **138**, 535-54.
- BARNES, H. (1954). Some tables for the ionic composition of sea water. *J. Exp. Biol.* **31**, 582-8.
- CHAMBERS, R. & POLLACK, H. (1927). Micrurgical studies in cell physiology. IV. Colorimetric determination of the nuclear and cytoplasmic pH in the starfish egg. *J. Gen. Physiol.* **10**, 739-55.
- HAGSTRÖM, B. E. (1956). Studies on the fertilization of jelly-free sea-urchin eggs. *Exp. Cell Res.* **10**, 24-28.
- HARVEY, E. B. (1956). *The American Arbacia*. Princeton, New Jersey: Princeton University Press.
- LASER, H. & ROTHSCHILD, LORD (1939). The metabolism of the eggs of *Psammechinus miliaris* during the fertilization reaction. *Proc. Roy. Soc. B*, **126**, 539-57.
- NERDHAM, J. & NERDHAM, D. M. (1926). The hydrogen-ion concentration and oxidation-reduction potential of the cell-interior before and after fertilization and cleavage: a micro-injection study on marine eggs. *Proc. Roy. Soc. B*, **99**, 173-99.
- ÖRSTRÖM, A. & LINDBERG, O. (1940). Über den Kohlenhydratstoffwechsel bei der Befruchtung des Seeigeleies. *Enzymologia*, **8**, 367-84.
- ROTHSCHILD, LORD & SWANN, M. M. (1952). The fertilization reaction in the sea-urchin. The block to polyspermy. *J. Exp. Biol.* **29**, 469-83.
- ROTHSCHILD, LORD (1953). The fertilization reaction in the sea-urchin. The induction of polyspermy by nicotine. *J. Exp. Biol.* **30**, 57-67.
- ROTHSCHILD, LORD (1956). *Fertilisation*. London: Methuen and Co. Ltd.

- RUNNSTRÖM, J. (1933). Zur Kenntnis der Stoffwechselfvorgänge bei der Entwicklungserregung des Seeigelleies. *Biochem. Z.* 258, 257-79.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Techniques*. Minneapolis, U.S.A.: Burgess Publishing Co.
- YČAS, M. (1950). Studies in the respiratory enzymes of sea urchin eggs. Ph.D. Dissertation, California Institute of Technology.

APPENDIX

*Relationship between acid production, as  $\mu\text{l. CO}_2$ , and manometer reading*

The following assumptions or definitions are made:

- (1)  $k_{O_2} = 1.40$  and  $k_{CO_2} = 1.63$ ,
- (2)  $\text{CO}_2(R) = \text{respiratory CO}_2$ ,
- (3) R.Q. =  $\text{CO}_2(R)/\text{O}_2 = 0.7$  (Laser & Rothschild, 1939),
- (4)  $\text{CO}_2(A) = \text{CO}_2$  due to acid production =  $+a \mu\text{l.}$ ,
- (5)  $\frac{\text{CO}_2(R) + \text{CO}_2(A)}{\text{O}_2} = \frac{130}{70} = 1.86$  (Laser & Rothschild, 1939).

Hence 
$$\frac{\text{CO}_2(R) + a}{\text{O}_2} = 1.86,$$

and 
$$\frac{\text{CO}_2(R) + a}{10\text{CO}_2(R)/7} = 1.86,$$

and 
$$7\text{CO}_2(R) + 7a = 18.6\text{CO}_2(R),$$

and 
$$\text{CO}_2(R) = 0.605a.$$

The manometer reading,  $H$ , will be

$$H = +\frac{a}{1.63} + \frac{0.605a}{1.63} - \left( \frac{10}{7} \times 0.605a \times \frac{1}{1.40} \right) = a/2.7.$$

The acid production, in  $\mu\text{l. CO}_2$  is therefore 2.7 times the observed manometer reading.

ADDENDUM

Aketa (1957, pp. 273-5), says there is a marked increase in lactic acid content following the fertilization of Japanese sea-urchins and that 'This observation conflicts with those of other workers, who have been unable to detect any such change'; and that the difference may be ascribed to the fact that 'other investigators performed their estimations at longer intervals after insemination than the author did'. Referring to the fertilization acid, Aketa says 'it is still premature to deny the possibility of a relation between the manometric production of unknown acid and the lactic acid production shown in this paper'. On the basis of these remarks, I concluded that Aketa believed the fertilization acid was lactic acid. But in correspondence since the acceptance of this paper, Aketa told me he did not believe the fertilization acid was lactic acid, but that it would be unwise to deny this possibility.