

THE FERTILIZATION REACTION IN THE
SEA-URCHIN

THE INDUCTION OF POLYSPERMY BY NICOTINE

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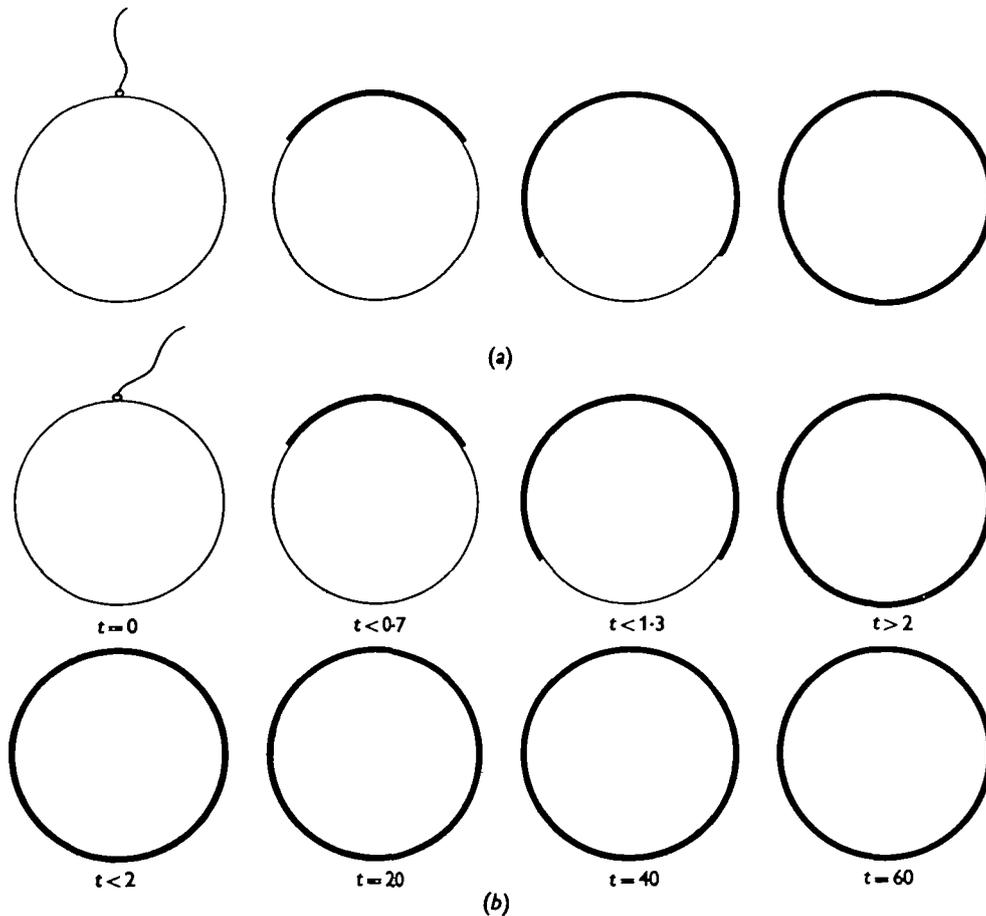
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(With Plate 5)

INTRODUCTION

Monospermy is one of the mechanisms which ensures that male and female hereditary characteristics are present in the offspring in approximately equal quantities. There are three main methods by which this is achieved: first, by the neutralization of supernumerary spermatozoa after they have entered an egg; secondly, by preventing supernumerary spermatozoa from entering an egg; and thirdly, by the death of a polyspermic embryo. The first of these methods has been observed in the eggs of birds, amphibians and possibly some mammals; the third requires no comment, while the second is the subject of this paper and may be defined as follows: when a spermatozoon fertilizes an egg a change occurs in the structure of the egg surface which prevents re-fertilization. This change in surface structure, the block to polyspermy, is initiated by the fertilizing spermatozoon and spreads over the egg surface from the point of sperm attachment. It may be likened to a skin which is impermeable to spermatozoa, spreading or expanding over the egg surface (Text-fig. 1 *a*). Recent experiments show that this model is over-simplified and that the skin, instead of being totally impermeable to spermatozoa (or 'black'), from the moment the fertilizing spermatozoon causes it to appear, starts by being relatively impermeable to spermatozoa (or 'grey'), as in Text-fig. 1 *b*. Later on, the egg surface becomes completely impermeable to supernumerary spermatozoa, when the block to polyspermy is complete. These conclusions about the block to polyspermy are based on a series of experiments (Rothschild & Swann, 1949-52) which are summarized immediately below. When a sea-urchin egg is fertilized, a change in the structure of the egg surface, the cortical change, spreads over the cortex from the point of attachment of the fertilizing spermatozoon. The conduction time of this change is of the order of 20 sec. Calculations, which involve treating spermatozoa as gas molecules bombarding a sphere (the validity of which is discussed later), show that if this cortical change is the block to polyspermy, the probability of a successful sperm-egg collision, in the sense that fertilization follows the collision, must be of the order of 1 in 160. This question was investigated in another series of experiments in which it was shown that though the probability of a successful sperm-egg

collision was markedly less than one, it was not low enough, i.e. $1/160$, to support the hypothesis that the cortical change was the block to polyspermy. At the same time, there was other evidence showing that the block to polyspermy was a comparatively slow reaction, taking something of the order of seconds to cover the egg surface. These apparent contradictions were resolved when the block to polyspermy was shown to be diphasic, in the sense that it consisted of a fast component



Text-fig. 1. *a*, simplified diagram of block to polyspermy. *b*, revised diagram showing rapid partial block (grey), and slow complete block (black). Time, t , in seconds.

with a conduction time of less than 2 sec. and a slow component which takes about 60 sec. to cover the egg surface and make it completely impermeable to spermatozoa. After the fast phase of the block to polyspermy is complete the chance of re-fertilization is reduced by a factor of about 20.

The fertilization rate or parameter, α . As there are several references to α in this paper, some observations on its significance may be desirable. No recondite arguments are needed to support the contention that the chances of fertilization depend, among other things, on the speeds at which spermatozoa swim, if only

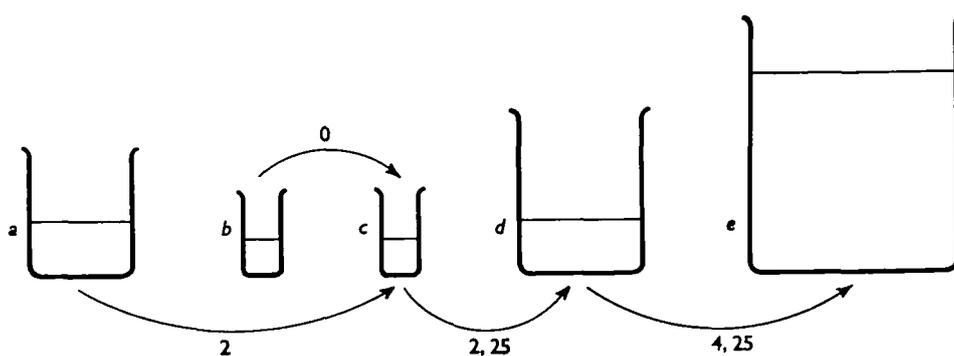
because it is easy to show that spermatozoa which are normally motile, but which have been rendered motionless, cannot fertilize eggs. It has, however, proved valuable to regard a mixture of eggs and spermatozoa as a system of spheres being bombarded by ideal gas molecules, moving at known speeds. The application of kinetic theory principles to suspensions of eggs and spermatozoa revealed, as mentioned above, that the conduction time of the block to polyspermy could not be of the order of 20 sec. unless the probability of a successful sperm-egg collision was 1 in 160 or less. Furthermore, this quantitative examination of the fertilization reaction enabled an estimate to be made of the probability of a successful collision. Some biologists have nevertheless expressed doubts about the validity of using concepts borrowed from the kinetic theory of gases as an aid to understanding the fertilization reaction. These doubts are based on the idea that the expression 'sperm-egg collision' implies that if we were to take a ciné-film of a single egg in a suspension of spermatozoa, we should see numbers of marble-like or, more accurately, tadpole-like objects colliding with and bouncing off the egg surface. It is hardly necessary to say that spermatozoa do not behave in this way: almost every student of the fertilization reaction must have noticed spermatozoa stuck to the surface of an egg but unable to fertilize it. Such deviations from 'ideal' behaviour are, in fact, unlikely to make estimates of collision frequencies seriously in error; but even if we do not start with the idea of sperm-egg collisions, we are inevitably driven to some such concept when investigating the relationship between the number of fertilized eggs in a suspension and the time of contact between the eggs and spermatozoa in that suspension. When this experiment is carried out, the curve relating the proportion of fertilized eggs to the sperm-egg interaction time is found to be of the form $f = 1 - \exp(-\alpha t)$, where α is a constant and t is the interaction time (maximum, 40 sec.). If, on the other hand, we make the plausible and weak assumption that the probability of an egg being fertilized in an interval of time δt is proportional to that interval of time, the proportionality constant being denoted by α , we again arrive at the equation $f = 1 - \exp(-\alpha t)$. For purely dimensional reasons, α must be a rate or frequency, because the exponent on the right-hand side of the equation must be dimensionless. No information is lost by considering α as a fertilization rate or parameter, but if this is held to be too abstract, α can be thought of as the product of the sperm-egg collision frequency, Z , and the probability of such a collision being successful, p . The α concept does not involve consideration of such questions as the random movements of spermatozoa, chemotaxis, or the trap action of jelly; these only arise when α is expressed in terms of Z and p .

Nicotine. Pre-treatment of eggs with nicotine increases the chance of re-fertilization by a factor of about 20, when insemination is carried out at high sperm densities 10^8 /ml. (Rothschild & Swann, 1950). The object of the experiments described in this paper was to find out how the nicotine treatment achieves this result. There are three or perhaps four possibilities, which could occur separately or in combination: (1) nicotine might increase the speeds at which spermatozoa swim, thereby increasing the probability of a sperm-egg collision; in other words it might increase α . (2) Nicotine might lengthen the conduction time of the block to polyspermy, thus

increasing the probability of supernumerary spermatozoa fertilizing the egg before its surface became totally impermeable. (3) Nicotine might make the surface of the egg more receptive to spermatozoa. This is equivalent to increasing α , but via the egg and not the spermatozoon. We can imagine that if the surface of an egg were magnified sufficiently, it would appear to be covered with a series of incomplete but identical jig-saw puzzles. If nicotine made the consistency of these puzzles more like rubber than wood, it is not difficult to conceive that a spermatozoon might find it easier to complete one puzzle and initiate fertilization. (4) The fourth and unlikely mode of action of nicotine arises from the observation, mentioned earlier, that the conduction rate of the block to polyspermy is markedly non-uniform, there being a fast component which probably covers the egg in less than 2 sec. and a slower component which makes the egg totally impermeable to spermatozoa. As the conduction time of the cortical change is unaffected by nicotine (Rothschild & Swann, 1950) and as the fertilization membrane appears at the normal time in nicotine-treated eggs, the possibility that nicotine retards the slow component is remote.

MATERIAL AND METHODS

The gametes of *Paracentrotus lividus* were used at room temperature, 15–17° C. The jelly was not removed from the eggs and was present at the end of each experiment. Eggs and spermatozoa were allowed to interact with each other for known periods of time, functional separation being effected by the hypo-hypertonic treatment described in detail by Rothschild & Swann (1951*b*). This treatment was



Text-fig. 2. Experimental procedure. The curved lines with arrows show which vessels are emptied into which. The numbers by the curved lines refer to the times of emptying, e.g. 2, 25 means that vessel *c* was emptied into vessel *d* 2 min. 25 sec. after the beginning of the experiment, which started when *b* was emptied into *c* at $t=0$. *a*, 10 ml. of sperm suspension; *b*, 2 ml. nicotine in sea water (1/1000, v/v); *c*, 2 ml. egg suspension; *d*, 90 ml. hypotonic sea water (28%); *e*, 700 ml. sea water + 21 ml. 11% NaCl in sea water.

particularly successful with the gametes of *P. lividus*, as the transfer of the eggs from one beaker to another did not cause any cytolysis, while the spermatozoa were killed by immersion in hypotonic sea water and no re-fertilization occurred on transfer to hypertonic sea water. The sequence of operations is shown diagrammatically in Text-fig. 2. Eggs were pre-treated with sea water containing re-distilled

nicotine (1/2000, v/v), for 2 min. before fertilization. The sperm-egg interaction time was 25 sec.

Although superficially this type of experiment appears to be simple, in practice a number of difficulties were encountered. These centred round the problem of adjusting four variables simultaneously to achieve 30–70% fertilization in the egg suspensions. The variables in question were: the concentration of nicotine in the sea water; the duration of the pre-treatment of eggs with nicotine—there is a non-linear strength-duration relationship which determines the incidence of polyspermy; the sperm-egg interaction time; and the density of spermatozoa in contact with the eggs. The only way of solving this problem was by undertaking a series of pilot experiments before each of the experiments referred to in the Results Section. It was impossible to standardize the experimental conditions once and for all because eggs differed in their susceptibility to nicotine from batch to batch, and the proportion of dead spermatozoa in a sperm suspension also varied in samples from different sea-urchins.

The usual controls for unfertilizable eggs, parthenogenetic activation and fortuitous fertilization at times other than 0–25 sec. were done as a matter of routine.

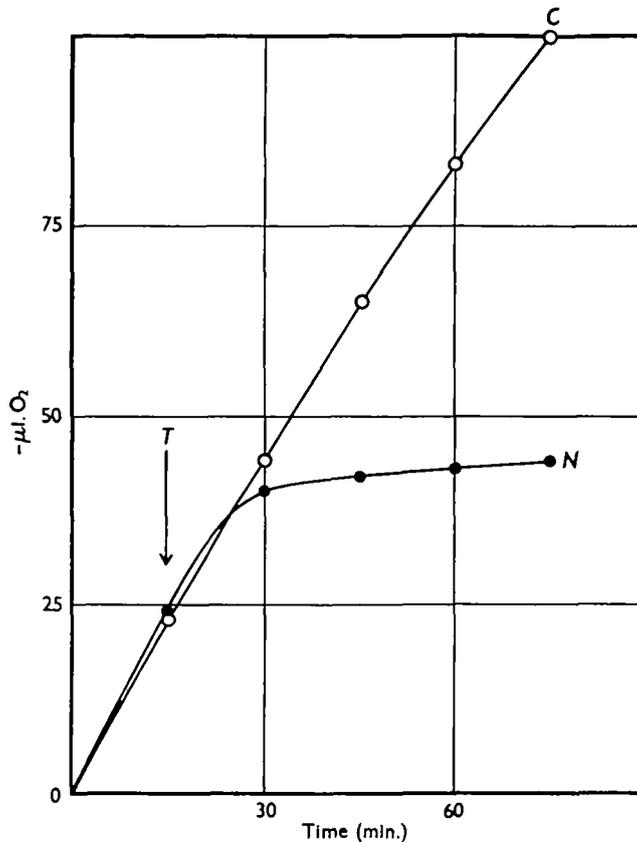
Measurements of sperm respiration were made in conventional Warburg differential manometers, by the direct method.

RESULTS

Effect of nicotine on spermatozoa. Visual examination showed that nicotine did not stimulate spermatozoa to increased activity, while dark ground tracks of spermatozoa in very dilute suspensions treated with nicotine confirmed the results of visual examination. These rough observations were amplified by measurements of the O_2 uptake of spermatozoa, before and after addition of nicotine to the medium (Text-fig. 3). Evidently nicotine is highly toxic to sea-urchin spermatozoa though there is a time-lag before it exerts its toxic effect. These experiments establish that nicotine does not induce polyspermy by increasing the activity of the spermatozoa, which could increase the chances of fertilization.

Probability of fertilization. Having disposed of the possibility that the activity of the spermatozoa is increased by nicotine, the eggs were tested to see if the nicotine altered their surfaces in such a way that the probability of fertilization was increased. Suppose that the sperm density in contact with the eggs and the sperm-egg interaction time are so adjusted that *not all* the eggs, but only a proportion, are fertilized at the end of the experiment. In these conditions, if nicotine were to increase the probability of fertilization, pre-treatment with nicotine should increase the proportion of eggs fertilized. The results of experiments to test this hypothesis are given in Table 1. The same number of eggs, pre-treated or not pre-treated with nicotine, were allowed to interact with the same number of spermatozoa for 25 sec. The proportion of fertilized eggs was determined after first cleavage in each case. Table 1 shows that nicotine does increase the probability of fertilization, α_N/α_C being greater than one in each case; but this increase, the average of which is 3.5, is

insufficient to account for the facts of polyspermy in treated and untreated eggs, the required factor being about 20. The eggs of *P. lividus* are similar to those of *Psammechinus miliaris* regarding the incidence of polyspermy in untreated eggs (Rothschild & Swann, 1950), which means that α is of the same order in both species.



Text-fig. 3. Oxygen uptake of sea-urchin spermatozoa after addition of nicotine. Contents of flasks, 3 ml. of semen diluted 1/100 with sea water. Side-arms, curve N, 0.3 ml. nicotine in sea water; curve C, 0.3 ml. sea water. T, side-arm contents tipped into main compartments of flasks. Centre wells, 0.2 ml. 10% KOH and filter-papers; shaker, 90 c.p.m.; stroke, 4.0 cm.; temperature, 17° C.; gas phase, air; vessel constants, app. 1.

During these experiments certain other observations were made and deserve mention:

(1) If an egg is fertilized just at, or before, the moment of transfer to hypotonic sea water, activation of the egg occurs but division does not take place. Membrane elevation is restricted to a small blister at the point where the sperm became attached to and activated the egg. The egg nucleus swells up and moves to the centre of the egg, but no development occurs. Except for the lack of division the phenomenon is reminiscent of the pseudogamy which occurs in *Rhabditis monohystera* and *R. pellio* (Peacock, 1944). Such activated eggs are not difficult to identify, provided it is realized that uncleaved eggs without two asters or a normal fertilization membrane

have been 'fertilized'. The investigation of this phenomenon, though of interest, is not relevant to the main subject of this paper.

(2) When doing this type of experiment it is important to try to arrange the experimental conditions so that the proportion of fertilized eggs lies between about 0.3 and 0.7. If very high or very low proportions are obtained, discordant results may be encountered because of the large sampling errors that occur when the number of fertilized or unfertilized eggs in the suspension is small. For example in the first experiment in this series, which was rejected for this reason, there were 220 unfertilized and six fertilized eggs in the control suspension. This made α_N/α_G too high to be credible, because of the low value of α_G in the controls.

Table 1. *Proportion of fertilized (f) and unfertilized (u) eggs of Paracentrotus lividus in a suspension of homologous spermatozoa of known density n*

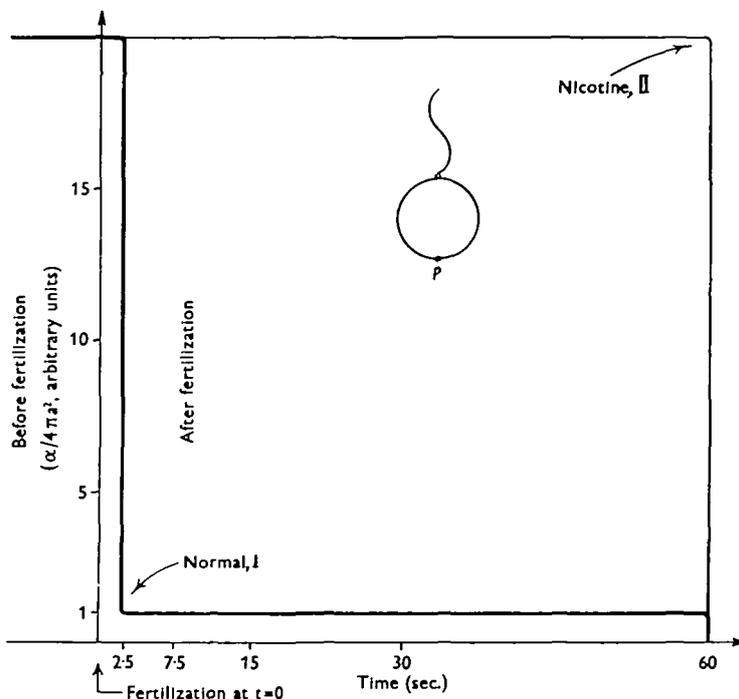
Sperm-egg interaction time, 25 sec. *E*, total number of eggs counted. *N*, eggs pre-treated for 2 min. with sea water containing nicotine (1/2000, v/v), *C*, controls, not pre-treated with nicotine. α is the slope of curve relating log *u* and *t*, in this instance 25 sec.

| Exp. no. | <i>n</i> | | <i>f</i> | <i>u</i> | <i>E</i> | α_N/α_G |
|----------|--------------------|----------|----------|----------|----------|---------------------|
| 1 | 1.06×10^8 | <i>N</i> | 0.274 | 0.726 | 299 | 1.2 |
| | | <i>C</i> | 0.228 | 0.772 | 180 | |
| 2 | 1.24×10^8 | <i>N</i> | 0.275 | 0.722 | 176 | 1.7 |
| | | <i>C</i> | 0.170 | 0.830 | 218 | |
| 3 | 1.88×10^8 | <i>N</i> | 0.239 | 0.761 | 431 | 1.8 |
| | | <i>C</i> | 0.144 | 0.856 | 278 | |
| 4 | 3.14×10^8 | <i>N</i> | 0.573 | 0.427 | 206 | 9.6 |
| | | <i>C</i> | 0.085 | 0.915 | 281 | |
| 5 | 5.24×10^8 | <i>N</i> | 0.737 | 0.263 | 224 | 4.0 |
| | | <i>C</i> | 0.281 | 0.719 | 228 | |
| 6 | 5.25×10^8 | <i>N</i> | 0.765 | 0.235 | 217 | 5.3 |
| | | <i>C</i> | 0.238 | 0.762 | 282 | |
| 7 | 5.57×10^8 | <i>N</i> | 0.840 | 0.160 | 263 | 4.3 |
| | | <i>C</i> | 0.350 | 0.650 | 309 | |
| 8 | 5.57×10^8 | <i>N</i> | 0.790 | 0.210 | 248 | 2.2 |
| | | <i>C</i> | 0.507 | 0.493 | 219 | |
| 9 | 5.63×10^8 | <i>N</i> | 0.425 | 0.575 | 214 | 2.6 |
| | | <i>C</i> | 0.191 | 0.809 | 199 | |
| 10 | 1.68×10^8 | <i>N</i> | 0.667 | 0.333 | 219 | 2.5 |
| | | <i>C</i> | 0.356 | 0.644 | 331 | |

(3) During these experiments it became clear that polyspermic eggs undergo their first cleavage markedly earlier than monospermic eggs. This phenomenon has interesting implications from the point of view of the mechanism of cleavage. As, however, it did not concern this investigation, the block to polyspermy in nicotine-treated eggs, the subject was not pursued.

(4) Reference has been made in this paper to unfertilized monospermic, polyspermic and activated eggs. All of these except the last have been repeatedly mentioned in previous papers. Pl. 5 *a* and *b* show how these different classes of eggs can be identified.

These experiments show without reasonable doubt that nicotine induces polyspermy by increasing the conduction time of the block to polyspermy and not by altering the egg surface in such a way that the probability of fertilization, i.e. α , is increased by the necessary amount. The probable forms of the block to polyspermy in nicotine-treated and untreated eggs are shown in Text-fig. 4.



Text-fig. 4. Curve I, variation in α per unit area ($\alpha/4\pi a^2$), at the point P on the egg surface, fertilization having occurred at the opposite pole at $t=0$. The first fall in α is due to the fast incomplete block to polyspermy reaching P . The second fall in α occurs when the complete block has covered the whole egg surface. Curve II, effect of nicotine on the block to polyspermy and therefore on α per unit area. The curves are, of course, idealized.

DISCUSSION

Time sequence of events. This is a convenient time to summarize our existing knowledge about the changes that occur in the sea-urchin egg immediately after fertilization. Contrary to earlier views, which in any case were not based on experimental evidence, the block to polyspermy is not an all-or-none process. After attachment of the fertilizing spermatozoon there passes over the egg surface a structural change which reduces the probability of re-fertilization by a factor of about 20. This change is completed in under 2 sec. At the end of it, however, the egg is not totally impermeable to supernumerary spermatozoa. Total impermeability is only achieved after some 60 sec., by which time the slow component of the block to polyspermy has completely covered the egg surface. The change in cortical structure which passes over the egg surface after fertilization, and whose conduction

time is about 20 sec., is clearly a reflexion of this slow component, though the time relationships of the whole event suggest that the fertilization membrane, or the processes immediately connected with its elevation, may well play a role, though perhaps a relatively minor one, in the prevention of polyspermy.* These facts can be expressed in a different and more quantitative way; when eggs are mixed with spermatozoa at a density of 10^6 /ml. the probability of a successful sperm-egg collision is about 0.05; that is to say, 1 in 20 collisions are, on the average, successful. After such a successful collision, the probability of a further successful collision, i.e. dispermy, falls to 1 in 400 in under 2 sec. At 60 sec. the probability of a successful collision falls to zero. Some confusion may have been caused by Just's remark (1939, p. 15) that 'in general, all normally monospermic eggs that I have studied are never polyspermic if they are in optimum condition'. This statement is of course incorrect. No mechanism could prevent polyspermy *ever* occurring because any change in egg structure must take a finite time to affect the whole of the egg surface. It therefore follows that under normal conditions of fertilization, polyspermy is not an impossibility but a rare event, the rarity depending on the speed at which the block to polyspermy covers the egg surface, that fraction of the total area of egg surface with which a spermatozoon can combine, the number of active spermatozoa in the neighbourhood of the egg in question, and the speeds at which the spermatozoa swim. When systematic counts are made of the incidence of polyspermy in egg suspensions which have been inseminated with spermatozoa at normal densities, the figures quoted above are found to be consistent with these counts. Naturally, the incidence of polyspermy goes down as the density of spermatozoa in contact with the eggs is reduced, for the simple reason that the lower the sperm density, the smaller the number of sperm-egg collisions that occur in a given time.

Polyspermy inducers. Treatments which cause polyspermy fall into two classes, over-insemination and chemical agents. Over-insemination is a method of forcing supernumerary spermatozoa into the egg both before the quick and slow blocks have covered the egg surface. The only chemical agent whose mode of action has been systematically investigated is nicotine, and the experiments described in this paper show that it induces polyspermy by increasing the conduction time of the block to polyspermy. The reasons for the view that this increase in conduction time is due to the abolition of the quick phase of the block have been given above. The question whether all chemical agents which induce polyspermy do so in this way cannot at present be answered. Some might well do so by the alternative method of increasing the probability of fertilization and therefore of re-fertilization. There is, at any rate, no structural similarity between several of the substances which induce polyspermy, though it is possible that the conduction mechanism is so delicately poised that interference with it can be achieved by non-specific means.

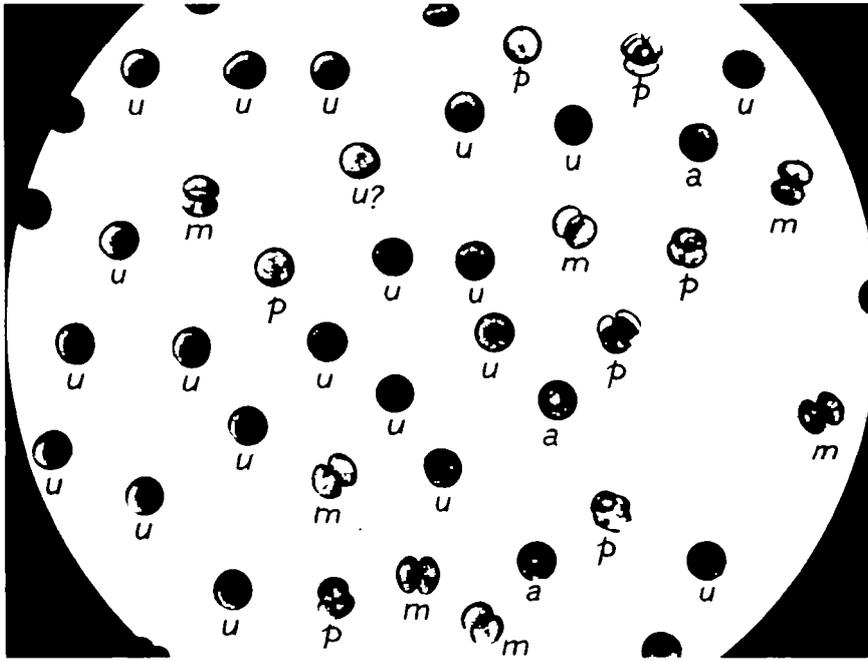
Mechanism of conduction. There has in the past been some discussion about the

* The conduction times of the cortical change and of the wave of exploding cortical granules described by Endo (1952) are sufficiently similar to make it very probable that these two phenomena are different aspects of the same process.

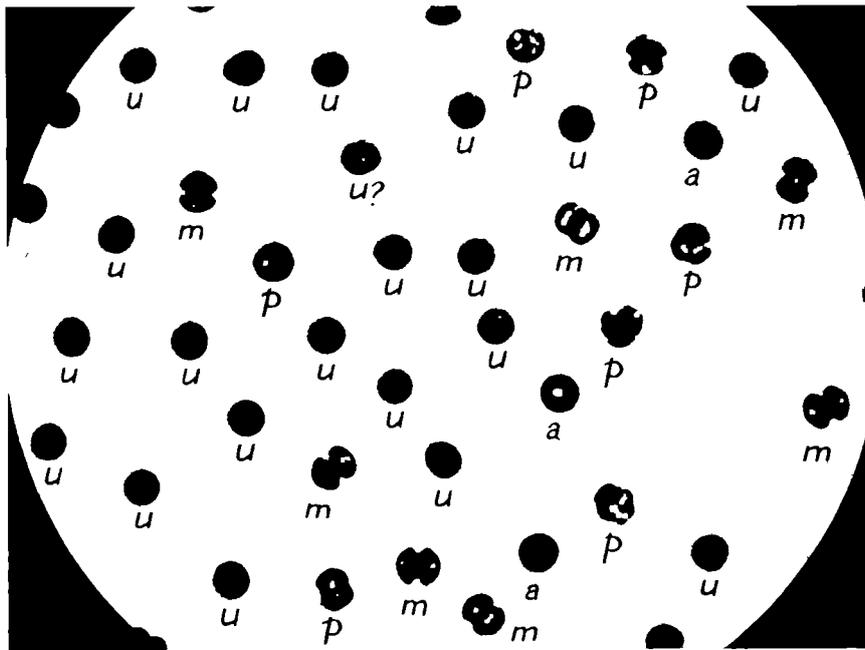
block to polyspermy being conducted through the egg cytoplasm or round the cortex. This question has become less important, if not academic, now it is known that the block to polyspermy has a fast component. Reference to the diffusion curves in the paper on this subject (Rothschild, 1949), apart from more general considerations, indicates that an intracellular diffusion mechanism is unlikely to account for this fast component. Recently, Runnström & Kriszat (1952) have published a paper in which they argue that the block to polyspermy is conducted round the cortex and not through the cytoplasm. This view is based on experiments involving the fertilization of damaged eggs. At fertilization, such eggs form unfertilized ex-ovates which can be fertilized later, as evidenced by the elevation of a fertilization membrane and a change in the consistency of the cytoplasm. These experiments probably confirm the view, expressed above, that the fast component of the block to polyspermy is a cortical reaction. As regards the slow component of the block, with which the visible change in the cortex is obviously connected, the position appears to the writer to be exactly as before, and unaffected by experiments on pathological eggs, which merely show that, as might be expected, maltreatment of an unfertilized egg damages the egg surface. There is evidence in this paper, e.g. pseudogamous fertilization, and in that of Runnström & Kriszat, to show that cytoplasmic changes occur after the attachment of the fertilizing spermatozoon to the egg surface. The time course of these changes is consistent with the hypothesis that the complete block to polyspermy is achieved by intracellular diffusion.

SUMMARY

1. Treatment of unfertilized sea-urchin eggs with sea water containing nicotine is known to induce polyspermy when the eggs are subsequently inseminated with homologous spermatozoa, at densities which cause a very small amount of polyspermy in untreated eggs.
2. If nicotine were to increase the speeds at which sea-urchin spermatozoa swim, the chances of fertilization, and therefore of polyspermy, might be increased. Nicotine does not increase sperm speeds; in addition, it causes a sharp reduction in the O_2 uptake of these spermatozoa.
3. The only other ways in which nicotine could induce polyspermy are by altering the egg surface in such a way that the probability of fertilization is increased by a factor of about twenty; or by lengthening the conduction time of the block to polyspermy. Experiments described in this paper show that the first explanation is untenable and therefore that the second is the correct one. It is concluded that nicotine abolishes the fast incomplete block to polyspermy and that over-exposure to this substance probably abolishes the block to polyspermy altogether.
4. Polyspermic eggs divide sooner than monospermic ones.
5. When, as in these experiments, eggs are allowed to interact with spermatozoa for known times, and then functionally separated by immersion in hypotonic sea water, some eggs, presumably those which sustain a successful sperm-egg collision at the end of the interaction time, are activated but not fertilized by the spermatozoon,



a



b

as in the pseudogamous nematodes. Cleavage does not occur though the egg nucleus swells.

6. Previous results in the same field and observations by other workers are discussed.

I am much indebted to the Director and Staff of the Zoological Station, Naples, for their kindness and hospitality, and to the Medical Research Council for the provision of a laboratory assistant.

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EXPLANATION OF PLATE 5

- (a) Suspension of eggs of *Paracentrotus lividus*, with jelly, showing *u*, unfertilized eggs; *m*, monospermic eggs; *p*, polyspermic eggs; *a*, activated eggs.
- (b) The same suspension deliberately photographed out of focus, to show *u*, eccentrically placed nuclei in unfertilized eggs; *p*, asters in polyspermic eggs which have failed to divide; *a*, central and enlarged female nuclei in activated eggs.
- Note doubtful unfertilized egg, *u*? Magnification, 54.