Table 2. Effects of diffusate and adenine on germinating seeds of lanceolate.

Normal	Lanceolate	Reduced	Modified	Total
		Diffusate		
15	25	4	6	50
		Adenine		
	39	8	3	50

Table 3. Effect of various adjuvants on reduced embryos grown in White's medium.

Treatment	Reduced	Modified	Total
(i) Strong diffusate	4	1	5
(ii) Weak diffusate	1	3	4
(iii) Adenine	2	3	5
(iv) Adenine + indoleacetic acid	2	3	5
(v) Indoleacetic acid	5	0	5
(vi) Distilled water (control)	5	0	5

upon the proper balance of adenine and indoleacetic acid (auxin).

Our first success in attempting to obtain growth in the reduced phenotype came by treating germinating seeds of lanceolate with diffusate and adenine; the seeds, of course, contained embryos in the proportion of 1 normal : 2 lanceolate : 1 reduced. Diffusate was prepared in the following manner: 500 normal tomato seeds were soaked in 20 ml of distilled water. After 3 days the water, with diffusible substances, was removed, and distilled water was again added. This procedure was repeated daily for a total of 4 days. The solution removed from the seeds was lyophilized to dryness, and the powder was dissolved in 10 ml of distilled water. Fifty seeds of lanceolate were germinated on filter paper in a Petri dish which contained the 10 ml of concentrated diffusate. Of the 50 resulting seedlings, four were typical reduced plants but six were a new phenotype, which we called "modified" (Table 2). Instead of remaining as a cylindrical mass of tissue, the hypocotyl broadened at the tip to form a structure resembling a cotyledon, at the base of which a bud developed that later grew into a shoot. The mature plant that developed from this shoot was very similar to the narrow phenotype, although the latter always had two cotyledons and a plumule to begin with. A second 50 seeds were treated with 40 parts per million of adenine sulfate; they produced eight reduced seedlings and three of the modified phenotype.

Tomato seeds usually complete their germination in 7 to 10 days; however, it was noted that the normal seeds from which the diffusate had been removed failed to complete germination. The roots developed almost at the normal rate, the hypocotyl elongated only slightly, but the cotyledons never emerged from the seed coat, nor did they show any sign of growth. Thus, a substance or substances required for early cotyledon and shoot growth may diffuse out of the germinating seeds. If the substance or substances are continuously removed from the seeds, normal growth will not take place.

In a second experiment seeds from selfed lanceolate plants were soaked in distilled water for 3 days, then dissected under sterile conditions. Embryos without cotyledons, presumably homozygous lanceolate, were selected. These were placed individually in test tubes on an agar surface. All of the test tubes contained White's medium (*6*) which had been autoclaved with 1-percent agar. To this basic medium the following substances had been added after sterilization by filtration: (i) strong diffusate (1 ml of concentrated diffusate—diffusate from 50 seeds); (ii) weak diffusate (½ ml of concentrated diffusate plus ½ ml of distilled water—diffusate from 25 seeds); (iii) adenine sulfate, 40 parts per million; (iv) adenine sulfate, 40 parts per million, plus indoleacetic acid, 1 part per million; (v) indoleacetic acid, 1 part per million; (vi) distilled water, which served as a control.

Again the modified phenotype appeared when the reduced embryos were treated with either diffusate or adenine (Table 3). It is clear that a substance (or substances) which diffuses out of normal tomato seeds stimulates the development of cotyledon-like structures and buds in the reduced embryos. It is possible that the poorer results obtained at higher concentration of diffusate may be due to toxic substances in the diffusate or to an imbalance of factors. Adenine appears to give the full effect of the diffusate, and its effectiveness is not enhanced by indoleactic acid. Embryos which develop in response to either adenine or diffusate continue to grow when transferred to soil and produce a plant very similar to narrow; that is, the phenotype modified is a phenocopy of narrow.

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Mitotic Arrest by Deuterium Oxide

Abstract. In marine invertebrate eggs, where cell divisions occur without growth, deuterium oxide produces arrest of, or serious delay in, mitosis and cytokinesis. All stages requiring assembly or operation of mechanical structures in the cytoplasm are sensitive to D_2O. The block is reversible in some cells.

Recent investigations of the effect of heavy water on biological systems have emphasized a finding implicit in the early papers—that in concentrations as low as 30 to 40 percent, D_2O arrests or delays cell division, without serious or immediate effects upon growth. Newer experiments on mammals and algae, respectively, are reported by Katz *et al.* (*1*) and by Moses *et al.* (*2*).

This result is of interest for two reasons: first, because the "isotope effect" is so large (see *3*), and second, because interference with cell division by so "simple" an agent offers some possibility of analysis in terms of primary molecular events.

We have undertaken a study of the effect of D_2O on fertilization and cleavage in the eggs of the sea urchin, *Arbacia punctulata,* and the annelid, *Chaetopterus pergamentaceus.* This report is a descriptive summary of the findings. Marine eggs were chosen for this study in order to avoid a source of confusion present in the systems heretofore investigated—that is, simultaneity of growth and division. As explained by Swann (*4*), division occurs in the absence of growth in the early development of the sea urchin. The same is true, presumably, for the egg of *Chaetopterus.*

Lucké and Harvey reported in 1935 (*5*) that cleavage of sea-urchin eggs was completely blocked in 99.5 percent D_2O, while in the same cells there was no evidence of rapid changes in permeability. Hoberman *et al.* (*6*) have found that cleavage is somewhat delayed in sea water containing a 9.1 atom-percent excess of D. Ussing (*7*), studying amphibia, and hampered by the necessity of employing minute volumes of medium, was able to show that even low concentrations of D_2O retard cleavage, and that no cleavage takes place in D_2O concentrations higher than 40 percent.

The experiments under discussion were concerned with the following variables: effect on fertilization compared with that on division, concentration of D_2O, stage of arrest or delay, possibility of reversal, and species.

When sea urchin eggs were inseminated in sea water and in a series of sea waters reconstituted with D_2O, it was found that eggs in media containing up to 25 percent (*8*) of heavy water fertilized normally. The criterion for

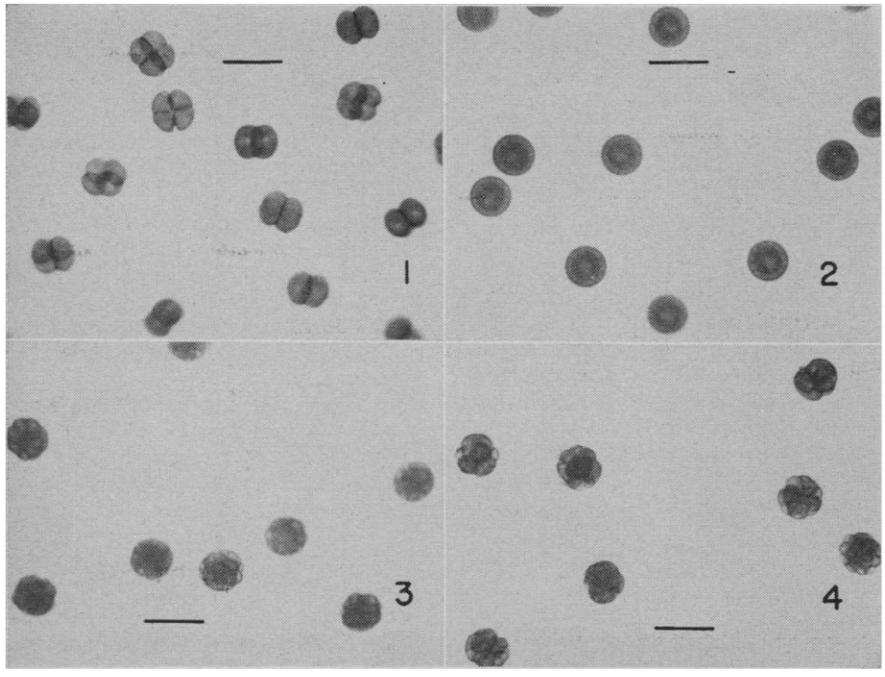

Fig. 1. *Arbacia* eggs (controls), 93 minutes after fertilization, showing four-cell stages. Fig. 2. Eggs from the same batch, placed in sea water containing D_2O 15 minutes after insemination, photographed at 96 minutes. Fig. 3. Controls 3½ hours after fertilization. Fig. 4. The same eggs as in Fig. 2, returned to normal sea water after 85 minutes in D_2O. Multiple irregular cleavages are evident. The horizontal lines represent 100 μ.

normal fertilization was the elevation of a clearly separated fertilization membrane within 5 minutes after insemination. At higher concentrations, the number of eggs fertilized declines sharply, so that in 50 percent D_2O, less than half of the eggs ever elevate membranes; in 75 percent D_2O, about 10 percent do so, and in 96 percent D_2O, 2 percent of the eggs are fertilized. Sperm motility seems to be unaffected, even in media containing 96 percent heavy water, for at least several minutes after dilution of the seminal fluid.

The effect of D_2O on cell division is separable from the effect on fertilization, as is shown in the following manner. Eggs were transferred from normal sea water, in which they had been fertilized, to sea water containing 96 percent D_2O, at 20, 30, 42, 47, and 50 minutes after insemination. Simultaneously, control samples were transferred to reconstituted sea water prepared from dried salts and distilled water. Mitotic events were followed by continuous observation of the cells and by photography of several low-power fields of each sample at intervals of about 5 minutes. All of the controls showed a normal sequence of events: the beginning of a "streak" at 20 minutes, metaphase spindles at 42 minutes, a few furrows beginning at 47 minutes (hence, anaphase), and normal cleavages at 50 minutes. The cells in D_2O were all arrested *at the stage of entry*. No cleavages (other than those already present in the 47- and 50-minute samples) were accomplished in these cells, even when the controls were entering the second division. Thus, it appears that the block occurs in all stages of mitosis and cytokinesis.

Sixty-six minutes after the immersion of each batch in D_2O, the experimental samples were washed with normal sea water. Their development was followed for several hours. Some reversal of the block was seen in all of the experimental samples; within 60 minutes after washing, all samples contained numbers of dividing cells. Some 30 to 40 percent of the treated cells never divided, but in the remainder, the divisions were frequently multiple and rapid, albeit irregular. There was a distinct tendency for treated cells to "catch up" with controls. This suggests that metabolic work and chromosome duplication were not blocked.

The photographs (Figs. 1–4) were made during another experiment in which the cells were immersed in D_2O fifteen minutes after insemination. Figure 1 shows the condition of the controls 93 minutes after fertilization, and Fig. 2, that of the D_2O-treated cells 96 minutes after fertilization. The blocked cells had formed no spindles, and the nuclear membrane had not broken down, but a curious mottling of the peripheral cytoplasm appeared. After 85 minutes in D_2O, the experimental cells were washed and returned to normal reconstituted sea water. Figure 3 shows the controls at 3½ hours after fertilization, and Fig. 4 shows the condition of the washed eggs at the same time. Evidently, a significant reversal of the block had been effected.

Experiments with lower concentrations of D_2O show that the effect of D_2O on division is more severe than the effect on fertilization. In sea water containing 75 percent D_2O, division remains completely blocked; in 50 percent D_2O, many eggs remain blocked and some cleave, but only after a delay of from three to four times the normal cleavage interval. Only below a concentration of 10 percent does the interference with cell division disappear.

Similar results were obtained with *Chaetopterus* eggs—that is, a 95-percent concentration of D_2O blocks cytokinesis and mitosis at all stages beyond early prophase. In this form, the number of cells ultimately dividing after removal from D_2O is much smaller than in *Arbacia*, but a degree of reversibility of the effect is evidenced by the dramatic disappearance of the gross shrinkage and "blebbing" seen during the D_2O treatment.

Thus it appears that the formation of normal mitotic spindles and cleavage furrows is impossible in concentrations of D_2O higher than about 75 percent. The data suggest that the primary effect is on processes associated with the mechanical structures of the dividing cell and on the assembly of these structures. This would imply much less interference with the metabolic events preceding, and necessary to, mitosis. The implication is supported by the observation of multipolar cleavages in the cells removed from D_2O after a long immersion. One might suppose the site of the isotope effect to be a large, extensively hydrogen-bonded structure, possibly the forming or complete mitotic apparatus itself (*9*).

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Nonlogarithmic Linear Titration Curves

Abstract. Titration curves can be based on linear nonlogarithmic forms of the equilibrium equation of a dissociation reaction. From such curves, in contrast to those based on logarithmic transformations, both the end point of the titration and the dissociation constant can be derived.

In recent articles in *Science* (*1*), graphic methods, based on a linear logarithmic transformation of the equation $(X) \cdot (Y)/(XY) = K$, have been recommended for the purpose of estimating the kinetic constants of dissociation reactions of the type $X + Y \rightleftharpoons XY$. However, the equilibrium equation can also be written in several nonlogarithmic forms, one of which is:

$$(P) = (X) + (XY) = (XY) + K \cdot (XY)/(Y) \qquad (1)$$

Thus, when X is titrated with Y, a plot of (XY) versus $(XY)/(Y)$ gives a straight line. The intercept with one of the coordinates equals P (the end point of the titration), while K is given by the slope. Linear logarithmic plots do not give the end point of the titration, which must be known for the construction of the curves. This is a disadvantage in cases where the concentration of the compound to be titrated is unknown.

Equation 1 is applicable to any dissociation reaction where the (relative) concentrations of XY and that of one of the dissociation products can be measured. For instance, it applies to the "titration" of an enzyme with its substrate, where the "end point" is most often unknown.

For this purpose, three nonlogarithmic linear equations were first suggested by Woolf (see *2*). The equation analogous to Eq. 1 and, for various reasons (*3*) to be preferred to the other two, can be written as:

$$(E_t) = (E) + (ES) = (ES) + K_M \cdot (ES)/(S)$$

or:

$$V_m = v + K_M \cdot v/(S)$$

where v (the initial reaction rate) equals $k \cdot (ES)$ and V_m (the maximal rate for $(S) \rightarrow \infty$ or the "end point of the titration") equals $k \cdot (E_t)$. Since, owing to low enzyme concentration, the concentration of free substrate is practically equal to the total substrate concentration (S), a plot of v versus $v/(S)$ is linear. Extrapolation to $(S) \rightarrow \infty$ gives V_m, while K_M (the Michaelis constant) is given by the slope.

A further example is the titration of the salt of a weak acid (AH) with a strong acid. From

$$(A^-) \cdot (H^+)/(AH) = K_H$$

it follows that

$$P = (A^-) + (AH) = (AH) + K_H \cdot (AH)/(H^+)$$

P is found by extrapolation of a plot of (AH) versus $(AH)/(H^+)$ to $(H^+) \rightarrow \infty$. K_H is derived from the slope of the plot.

When the sodium salt of AH is titrated withHCl, one has on the basis of electroneutrality (*4*):

$$(A^-) + (Cl^-) + (OH^-) = (Na^+) + (H^+)$$

Furthermore

$$(A^-) + (AH) = (Na^+)$$

Thus

$$(AH) = (Cl^-) - (H^+) + (OH^-)$$

where (Cl^-) can be expressed in equivalents of added acid.

In Fig. 1 such titration curves are given for 10 ml of $10^{-2}M$ solutions of Na_2HPO_4 and of the sodium salt of diethylbarbituric acid (Veronal) titrated with $1N$ HCl by means of a micrometer and syringe with capillary tip. The pH measurements were carried out with a Radiometer pH-meter standardized with a buffer based on U.S. National Bureau of Standards certified buffers.

Since the smallest increment of HCl added constituted a concentration of (Cl^-) of $10^{-3}M$ in the reaction mixture, (H^+) and (OH^-) could be neglected over the entire range of pH values ($\sim$ 6 to 9) used, and the equivalents (T) of added HCl were simply plotted versus $T/(H^+)$. The slopes of the curves correspond to pK_H values of 7.02 and 7.90 (30°C, 0.01M ionic strength) for phosphate and diethylbarbituric acid, respectively.

When a solution of the acid AH is titrated with a strong base, for example NaOH, one finds that:

$$(AH) = (P) - (Na^+) - (H^+) + (OH^-)$$

that is, for a plot of (AH) versus $(AH)/(H^+)$, the end point must be known. However, since Eq. 1 can be written as:

$$P = (A^-) + (A^-) \cdot (H^+)/K_H$$

a plot of (A^-) versus $(A^-) \cdot (H^+)$, where

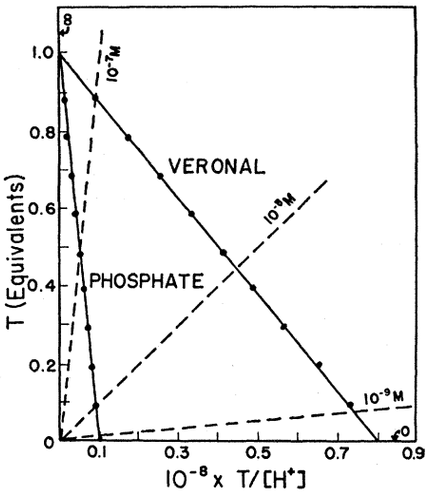

Fig. 1. Nonlogarithmic linear titration curves of phosphate and diethylbarbiturate (Veronal). T represents equivalents of HCl added to a solution of the sodium salt. $[H^+]$ is the hydrogen ion concentration. Points on the curves referring to a particular hydrogen ion concentration lie on a line through the origin. The ordinate represents $[H^+] = \infty$ and the abscissa $[H^+] = 0$. The intercept of the curves with the ordinate gives the end point of the titration, while the (negative) slope is equal to the hydrogen ion dissociation constant.

$(A^-) = (Na^+) + (H^+) - (OH^-)$, gives P as well as K_H, also in this case. The equations can readily be adapted to the case of a weak base.

This method of plotting titration data has the further advantage over linear logarithmic plots of being more sensitive to experimental errors and small deviations from theory. This is emphasized by the fact that for K_H values that differ by more than one pK unit, the scale of the plot must be adapted to each constant separately. On the other hand, a more detailed analysis of a composite titration curve, involving two or more K_H values that are less than one unit apart, can be made on the same scale by using the procedure described here (*5*).

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