MITOSIS AND MACROMOLECULE SYNTHESIS IN CELLS EXPOSED TO D_2O

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INTRODUCTION

Substitution of deuterium for hydrogen in the medium bathing a cell has many interesting consequences: the isotope effects for a number of processes can be very large indeed, compared with those measured in purely chemical or biochemical systems *in vitro*[1]. Among the most sensitive processes is mitosis: under suitable conditions, the effect can be infinite, *i.e.*, the cells never divide, and are held in a "frozen" condition so long as the deuterium remains[2].

The origin of this effect is obscure, and its analysis is made more difficult by the fact that a phylogenetic discontinuity exists: many micro-organismal cells can be adapted to grow in deuterium-enriched media[1], whereas cells of higher plants and animals have not thus far proved adaptable. This probably reflects fundamental differences in the mechanism of mitosis, as is also suggested by the cytologic details. One proposal, made a few years ago by Gross and Spindel[2, 3], continues to account well for the observations made on higher cells: it suggests that cytoplasmic structures associated with mitosis are stabilized, or "frozen", in the presence of heavy water, as a result of a co-operative effect on the hydrogen-bond crosslinks of macromolecular gels. Proof of such a hypothesis is difficult to obtain, however, especially as data from simpler systems, such as proteins and nucleic acids in solution and in the solid state, give contradictory evidence concerning the direction of a change in hydrogen bond strength.

The problem is worth pursuing, especially from a biological point of view. Understanding the mechanism of mitotic blockade by heavy water will add greatly to our knowledge of the organization and function of the mitotic apparatus. There are, moreover, two additional effects of heavy water which seem related to the antimitotic action; blockade of DNA synthesis[4], and the parthenogenetic activation of unfertilized eggs[5]. Special interest in the latter effect arises from the discovery that deuterium parthenogenesis is accompanied by release of the normally blocked protein synthesis in unfertilized eggs. Analysis of this phenomenon promises to be of material assistance in determining the nature of the control mechanism that operates upon protein biosynthesis at the beginning of embryonic life.

In what follows, we shall summarize first the present position concerning

the antimitotic effect, following which, experiments with gelatin solutions and gels will be described. This is a model system for the large changes in the mechanical properties of the cytoplasm which follow upon deuteration. The observations on deuterium parthenogenesis will then be described, together with a summary of the experimental data concerning protein synthesis and its uncoupling from RNA synthesis. Finally, an attempt will be made to bring the experimental data within the framework of the general descriptive hypothesis.

THE DEUTERIUM EFFECT ON MITOSIS AND ON THE CYTOPLASM

Some essential features of the antimitotic effect are represented in *Figure 1*. The data were obtained from a population of sea urchin eggs (*Arbacia punctulata*) fertilized at 20°. One half of the eggs was centrifuged and replaced in an artificial sea water containing 96 per cent D_2O at 15 minutes post-fertilization. The controls remained in normal sea water. Two complete

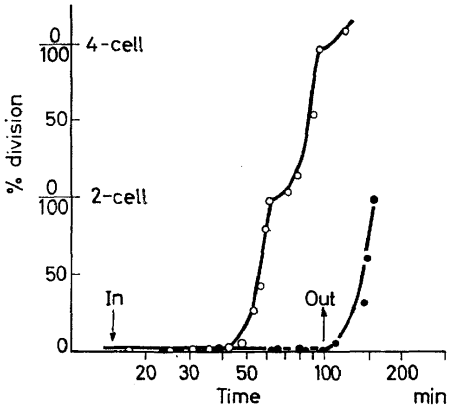

Figure 1. Reversible inhibition of cell division in sea urchin eggs by D_2O; 96% D_2O-sea-water substituted for normal sea-water medium (filled circles) at 15 min post-fertilization; times of addition and removal of D_2O shown by arrows; controls (open circles) complete two division cycles; (replotted from ref. 2)

cleavage cycles and the beginning of the third are represented as rising "percentage division" counts on the graph. The deuterated eggs show no cleavages whatsoever. At 100 minutes post-fertilization (when controls were dividing from four to eight cells), D_2O was removed and replaced with H_2O in the experimental suspension. After a delay of 20 minutes, these eggs began to divide. The divisions were multiple, *i.e.*, each egg divided not into two, but into several unequal blastomeres. Cytologic observation of the eggs while in D_2O reveals that they remain in the mitotic stage at which they entered heavy water. The experiment shown in *Figure 1* demonstrates the reversibility of the effect.

These earlier experiments[2, 3] on the cleavage cycle also gave evidence of the high speed of the effect: blockade of division occurs at all stages of the cycle (except possibly the last stages of cytokinesis), and is imposed almost instantaneously. This has been confirmed in temperature–pressure experiments reported by Marsland and Zimmerman[6]. Tucker and Inoué[7] have shown by an ingenious sedimentation method that a 30 per cent exchange of D_2O and H_2O across the cell membrane is accomplished within 2 to 3 seconds.

The proposal that deuteration of dividing cells stabilizes the mitotic apparatus has likewise been confirmed by these two groups. Inoué and co-workers[8], in particular, have observed that deuteration of a dividing egg causes a significant rise in the birefringence of the mitotic spindle. These changes are closely parallel to those observed upon changing the temperature in normal sea water.

When the concentration of D in the medium is below 50 per cent (approx.), cells continue to divide, but at reduced rates; the degree of inhibition increases exponentially with the concentration. Temperature-dependence of the mitotic inhibition (as distinct from *blockade* at high D_2O concentration) is indicated in the data of *Figure 2*. Here, the eggs were exposed to 30 per

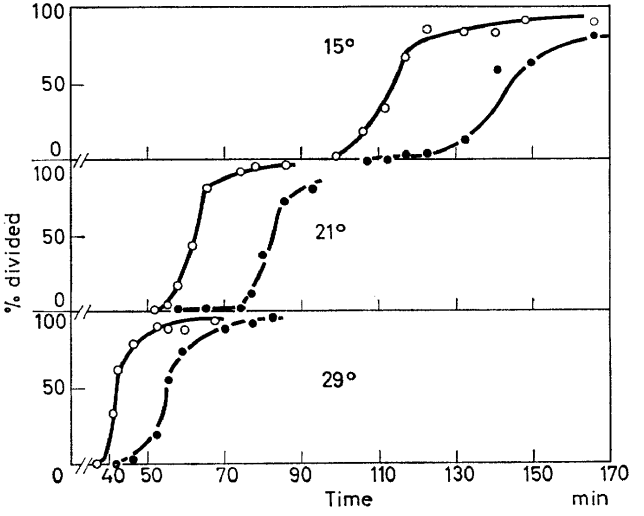

Figure 2. Patterns of cleavage in normal sea-water (open circles) and in 30% D_2O at different temperatures

cent D_2O-sea-water from the time of fertilization. It can be seen that the absolute length of the cleavage delay varies inversely with temperature. The normal cleavage rate, of course, increases with temperature, and in the range studied, has a heat of activation of about 13,000 cal/mole (this is in good agreement with earlier measurements of the "critical thermal increment" for cleavage[9]). Since the relative lengths of the cleavage cycle

and the D_2O delay remain unchanged, it may perhaps be inferred that the deuterium slows a process which is normally rate-limiting in this temperature range. The speed of the deuterium effect and its other characteristics argue strongly that beyond prophase this is not a metabolic or biosynthetic process, but rather one related to the mechanical properties of mitotic organelles.

That large changes in the mechanical properties of the cytoplasm as a whole can be evoked by deuteration of the cells is shown in *Figure 3.*

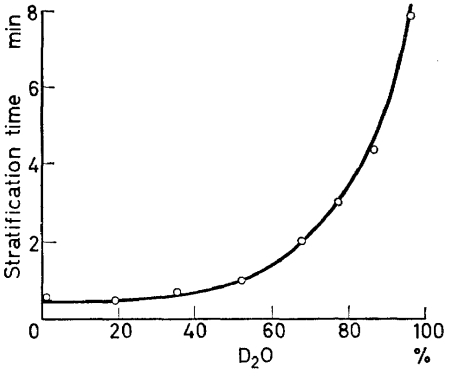

Figure 3. Effect of varying concentrations of D_2O on mechanical properties of the cytoplasm in unfertilized *Arbacia* eggs; stratification time is the period of centrifugation at 2000 × g (21°) required to produce a definite "clear zone" at the centripetal pole of 50% of the eggs

In the experiment, *unfertilized* eggs were placed for 30 minutes in sea-waters of increasing D content. Samples were then centrifuged. The larger cytoplasmic particles of the egg stratify under centrifugal force: in *Arbacia* eggs, a good standard endpoint is the time required, at a given centrifugal force, for a definite clear zone to appear at the centripetal pole. This is due to flotation and sedimentation of the fat droplets and yolk, respectively, in that region of the cell. The stratification time is an indirect measure of the mechanical rigidity of the ground cytoplasm. Arguments concerning the correctness of interpreting such measurements in terms of a "viscosity" are reviewed by Heilbrunn[10], but need not concern us here. The plotted data show that whatever property determines the stratification time (and it is probably related to the number and strength of the crosslinkages between soluble macromolecules in the ground cytoplasm), increasing the deuterium content of the cell's water (and of at least the exchangeable sites on the macromolecules) evokes an exponential increase in that property. The viscosity of the water is not alone determining: pure heavy water has a viscosity only about 20 per cent higher than that of H_2O, while the rigidity of the cytoplasm has increased at least sixteen-fold.

MODEL EXPERIMENTS WITH GELATIN

Studies on intact cells such as those described above suggest that the primary antimitotic effect is a mechanical one; perhaps a direct change in

the equilibrium properties of protein and nucleic acid components in organized cytoplasmic structure, such as the mitotic spindle and the chromosomes. The arguments centring round this interpretation have been summarized earlier[3]. Such changes might occur if upon substitution of D for H in the carboxyl, amino, and hydroxyl groups of amino-acid side chains in proteins, intermolecular hydrogen bond crosslinks were made slightly stronger (relative to H-bonds between these sites and solvent molecules).

A number of systems has been studied whose properties bear upon such a proposal, but their results have been contradictory. Data obtained from experiments in deuterated polypeptides and nucleic acids will be discussed below. It seemed useful, however, to attempt the study of a more relevant model, *i.e.*, one in which structure-formation is known to take place by the crosslinking of macromolecules in solution, largely or exclusively through hydrogen bonds.

Gelatin gels provide such a model, but they have two serious faults: First, gelatin is a poor model of cytoplasm, for, among other things, its gelation is exothermic and exhibits a volume decrease, while over the temperature range in which D_2O studies have been done with intact cells, the cytoplasmic gelation processes are endothermic[11] and have a positive ΔV (*i.e.*, they are inhibited or reversed by increase in hydrostatic pressure). Secondly, the properties of gelatins are poorly reproducible and depend upon the thermal history of the sample. These problems have been thoroughly reviewed by Ward and Saunders[12]. Yet gelatins provide a system in which structure forms rapidly, as it does in cells, and in which it is very likely that the predominant crosslink is the hydrogen bond. This appears to be a consequence of the structure of the parent collagens, from which gelatin is made: gelation probably represents the re-formation of the H-bonds which held together the native collagen triple helix, but now in a disordered way, and with a high proportion of inter-, rather than intramolecular links (see various authors, for example, in ref. 13).

The special problem of interpreting data on gelatins dissolved in D_2O is that the effects of heavy water upon the ionizations of the protein and the buffer salts can be estimated only inexactly, and gelatins are very sensitive to changes in pH and ionic strength. In order to deal with this, we adopted the strategy used by Laser and Slater in their studies on the inhibition of succinate oxidation by heart-muscle homogenates in heavy water[14]. This consists in performing all experiments at a number of values of pH, on both sides of the optimum. If the deuterium effect is largely or completely independent of pH (or pD), then difference in the behaviour of the water itself or of the electrolytes dissolved therein are not responsible.

Each of the types of experiment described below was done at several pH values, on both sides of the isoelectric point of the gelatin; also, the ionic strength was varied systematically. The qualitative differences between gelatins in D_2O and H_2O described here are therefore independent of such changes.

The gelatin was a highly purified calfskin product (batch 70-603) sold by Eastman Organic Chemicals. Its ash content was 0·03 per cent, the isoelectric point 4·9, the viscosity 140, and the jelly strength 370. Viscosities

of dilute solutions were measured in matched Ostwald-Fenske viscometers. Bath temperature varied by no more than 0·01° from the setting, and control and deuterated samples were always run side-by-side. Melting points were determined for gels by the method of Saunders and Ward[15]. All experiments were performed upon samples which were initially warmed to 60° and held there for one hour to erase the thermal history. In the heating experiments, the bath temperature was raised by 0·1°/min.

The variation in melting point with deuterium concentration in the solvent is shown in *Figure 4*. These data were obtained for a 1·67 per cent

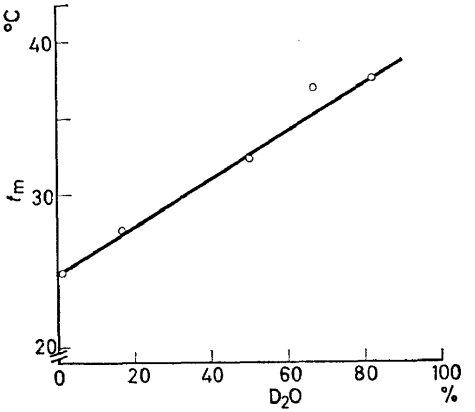

Figure 4. Variation of melting point of a 1·67% gelatin gel (containing 0·67M NaCl, pH in zero D_2O = 6·8) with concentration of D_2O in the solvent. Heating rate: 0·1°/min; melting temperature determined by fall of a CCl_4 droplet at the interface between gel and test-tube

gelatin gel, containing 0·67M NaCl, at pH (in the control) 6·8. It will be seen that the melting points rise by about 13° as the deuterium in the solvent is increased from effective zero to 83 per cent.

Data plotted in *Figure 5* show the effect of deuterium on the melting point

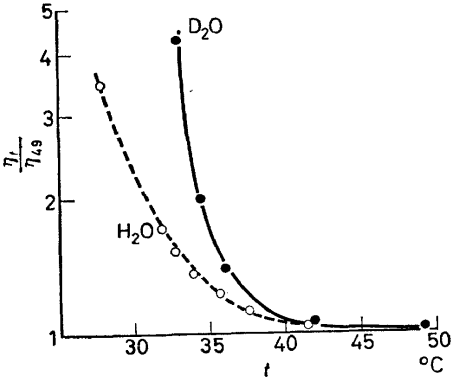

Figure 5. Ratio of specific viscosity at various temperatures to that at 49°, for gelatins in H_2O and in 83% D_2O; 1·5% gelatin, 0·67M NaCl, pH (in H_2O) = 6·3; semi-logarithmic plot

and on specific viscosity at temperatures above the melting point. Two gelatin solutions were prepared at a concentration of 1·5 per cent, with 0·37M NaCl, and at a pH (in H_2O) of 6·3. These were first heated to 60°, then left in a thermostat for 18 hours at 21·8°. At the end of this period, the deuterated sample was a gel, and the protonated one was a solution of moderate viscosity. The bath temperature was now raised very slowly, and viscosities measured as a function of indicated temperature. Once the D_2O gel had melted, the samples were always sheared together and the same number of times. In *Figure 5*, the ratio of specific viscosity at a given temperature to that at 49° is plotted against temperature. At 49°, the specific viscosities of the deuterated and control samples were equal. The data show that in addition to raising the melting point, deuteration of the solvent increases the solution viscosity, but that the differences are rapidly reduced as temperature is raised through and beyond 40°.

Figure 6 shows, finally, how the *reduced* viscosities of gelatin solutions

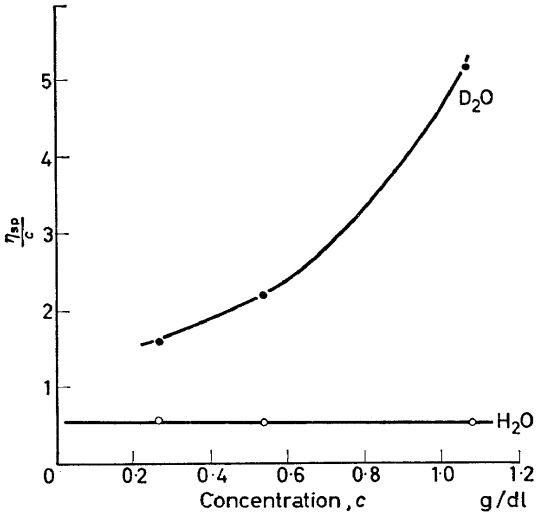

Figure 6. Reduced viscosities of gelatin in D_2O (filled circles) and H_2O (open circles) as a function of concentration of gelatin. pH in the H_2O-solution at highest concentration shown: 4·8 0·1M acetate buffer; plotted points are means of ten measurements

respond to the addition of deuterium to solvent. In these samples (at pH = 4·8 in the protonated solvent), the controls behaved normally, since the reduced viscosities did not vary greatly with concentrations, even at moderately high concentrations (but still well below the gel point at this pH). The D_2O samples, however, behave quite abnormally, showing a steady increase in reduced viscosity with concentration even at low concentrations, and higher values of reduced viscosity than in the controls at all concentrations. The intrinsic viscosities, however, which would be obtained by extrapolating to $c = 0$, are clearly not very different. This is evidence that the increments are not due to expansion or other shape changes of individual gelatin molecules in D_2O, but rather to a greater capacity for aggregate

formation at any concentration. The incremental capacity for aggregate formation is completely abolished when 8M urea is present, in which circumstances the reduced viscosity in D_2O is slightly, but significantly *lower* than that of gelatin in H_2O. These results seem consistent with the idea that a slight change in the strength of a D-bond, compared with an H-bond, can, through the co-operative interactions of many such bonds distributed along a polymer chain, cause a large net shift in the mechanical properties of the solution or gel, in the sense that intermolecular crosslinks become favoured over hydrogen bonds to solvent.

MACROMOLECULE SYNTHESIS AND DEUTERIUM PARTHENOGENESIS

It is surprising, in view of its unusual speed and effectiveness as an inhibitor of cell division, that D_2O is also an efficient parthenogenetic agent. The conditions under which artificial activation of the egg can be obtained with deuterium treatment are therefore of interest. The treatment schedule and some of the microsopic events associated with deuterium parthenogenesis are presented diagramatically in *Figure 7*. If an unfertilized egg is

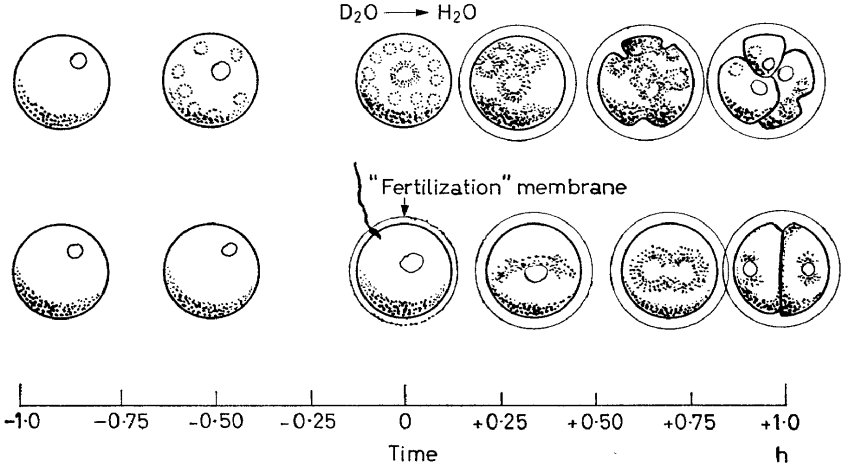

Figure 7. Schedule and results of parthenogenetic treatment for *Arbacia* eggs. Top set shows appearance of cytasters at +30 min of storage in D_2O, activation (lifting of membrane) just after transfer to normal sea-water, early multiple furrowing response at +30 min in normal sea-water, and multiple divisions, with formation of several true nuclei, at one hour; bottom set is corresponding schedule for unfertilized eggs (−1·0 to 0) and normally fertilized eggs in H_2O–sea-water; "streak" stage at +28 min, metaphase figure at +40 min, and normal first cleavage at +55 min

placed in heavy water (90 per cent D_2O-sea-water), cytasters appear in the cytoplasm. The number increases with time. If, after an hour's immersion in D_2O, eggs are transferred to normal sea-water, a high proportion (80 per cent of the population) raise a "fertilization" membrane, which gives evidence that activation has occurred. The cytasters now decrease in number, but those remaining enlarge greatly and are more like true mitotic

asters. At the periphery of the cell, they tend to associate in pairs. Within 35 minutes (at 21°) furrows are seen in the eggs, usually dipping down between pairs of cytasters lying just below the cortex. By the time normally fertilized controls complete their first cleavage, the deuterium-treated eggs have divided into several blastomeres. This sort of irregular and rapid cleavage continues for at least six hours, at the end of which the "embryos" are multicellular, but completely disordered.

When normally fertilized eggs are blocked in division by deuterium, working as an antimitotic, cytasters are also produced in large numbers. Earlier experiments with isotopically-labelled amino-acids showed that the cytasters are heavily labelled[16], and this, in combination with other evidence, suggested that the assembly of cytasters or of the normal mitotic apparatus requires new protein synthesis. This idea is supported by experiments of Hultin[17], which show that both protein synthesis and mitosis (and in particular, the formation of the mitotic spindle) are inhibited by puromycin, which prevents the attachment of amino-acids to peptide chains growing on the ribosome-messenger RNA complex. Now the unfertilized egg normally makes little or no protein, while fertilization releases the system within a few minutes, so that a very high rate of assembly of new proteins is attained early in the first division.

The appearance of cytasters in unfertilized eggs stored in deuterium, followed by very rapid cell division after return to normal sea-water, suggested that the deuterium treatment might be permitting the unfertilized egg to bypass whatever block exists for protein synthesis. The normal mechanism of the postfertilization release has been a matter of dispute. Some types of evidence, particularly from *in vitro* amino-acid incorporation systems, can be interpreted in favour of the idea that it is synthesis of new messenger RNA, after fertilization, that controls the "switching on" of protein synthesis[18–20]. Other evidence, including the behaviour of eggs treated with actinomycin[21], and the behaviour of parthenogenetic merogones[22, 23], suggests that the messengers or templates, are stored in some inactive form in the cytoplasm of the unfertilized egg.

The unfertilized egg is not only inactive in protein synthesis: it also makes little or no RNA[24], and hence can be assumed not to be "reading" its genes. We know, furthermore, that in the normally fertilized egg, D_2O treatment blocks DNA synthesis, depresses RNA synthesis, and has only a small effect upon protein synthesis[25]. It therefore seemed possible that in deuterium parthenogenesis, the appearance of cytasters during the period of D_2O storage might signal an uncoupling between protein synthesis and RNA synthesis. We now know that this is indeed the case[5], and some representative pieces of evidence will be cited.

A suspension of unfertilized eggs was divided into two; the cells were centrifuged and resuspended in artificial sea-water and in artificial D_2O sea-water (90 per cent), for controls and experimentals, respectively. These suspensions were at a cell density of 6×10^4 ml^{-1}, and contained valine-1-^{14}C at 1 μc/ml and 6 mc/mmole. The suspensions were sampled at intervals and the incorporation of labelled amino-acid into protein determined by counting in a low-background system at an efficiency of 10 per cent. Details of the preparation and counting techniques are given in earlier papers[5].

At two hours after immersion in D_2O, both these cells and the controls were recentrifuged and resuspended in normal sea-water, again containing ^{14}C-valine, as above. Sampling was continued for both suspensions. No activation was observed in the controls, but in the deuterium-treated eggs, 80 per cent showed at least one cleavage furrow within 35 minutes of their return to normal water.

Counting data are presented in *Figure 8*, in the form of differences in

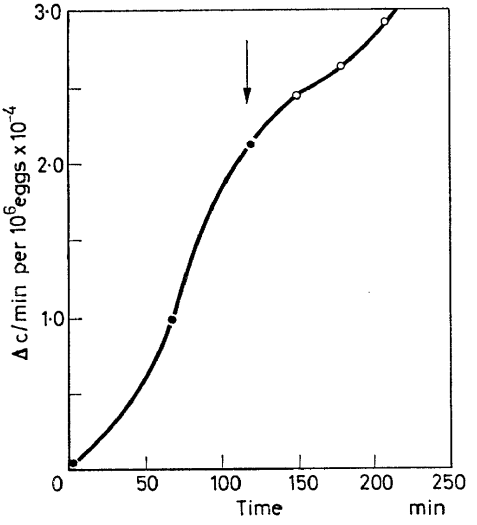

Figure 8. Differential incorporation of valine-1-^{14}C into proteins of eggs stored in D_2O–sea-water and in normal sea-water; cells washed and replaced in normal sea-water at the arrow (2 h); for details of biochemical preparations and radiation counting, see ref. 5

counts/min. between the experimental and control samples for 10^6 eggs. Since the incorporation into controls was small (see also below), these data represent a significant activation of protein synthesis (incorporation of labelled amino-acids measured by the techniques employed is a measure of protein synthesis[5]) *while* the eggs were still in heavy water. As can be seen in the figure, this activation survives removal of the heavy water; indeed, the rates are enhanced at that time.

Additional information is obtained from experiments such as that represented in *Figure 9*. Here, there were two controls: one suspension which remained unfertilized after removal of the D_2O from the experimentals, the other inseminated at that time, in order to compare the rates of protein synthesis in D_2O-activated eggs with those in normally fertilized ones. Cell densities here were 2×10^4 ml^{-1}, and the ^{14}C-L-valine was present continuously at 0·5 μc/ml and 10 μg/ml. It will be seen that as before, the controls incorporate only slightly (and even this is probably due to contamination of the culture with a few immature oocytes) during the period of D_2O storage in the experimentals. A high rate of synthesis is attained in the parthenogenetic eggs after removal of D_2O, even as compared with that

in normal fertilization. Other, later experiments show that this D_2O-induced protein synthesis continues for about ten hours at 21°.

During the period of D_2O storage, there is no incorporation of ^{14}C-thymidine into DNA, a not unexpected result, since such incorporation is blocked by heavy water even in normally fertilized embryos. After washing-out, a

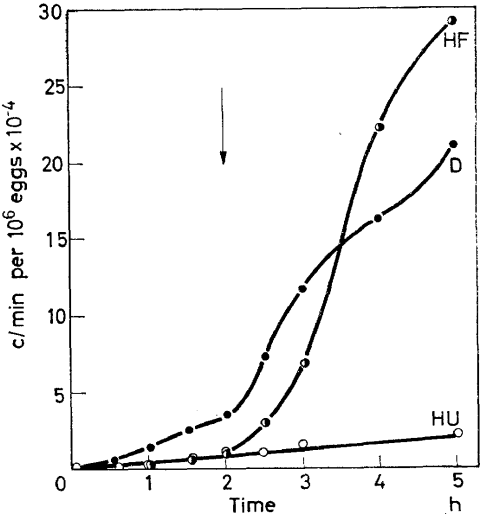

Figure 9. Incorporation of L-valine-1-^{14}C into proteins; filled circles: eggs preincubated in D_2O for 2 hours and then returned to normal sea-water; open circles, unfertilized eggs, stored in normal sea-water and returned to normal sea-water; circles with half fill: eggs stored unfertilized in normal sea-water for 2 h, then resuspended in normal sea-water and fertilized at two hours; arrow is at the transfer time for all cultures

rapid synthesis of DNA begins in the artificially activated cells[25]: the bulk counting and autoradiographic data from these experiments are relevant to the function of cytasters and will be dealt with in detail elsewhere.

The synthesis of RNA is for our present purposes, more relevant. It is known from the experiments of Cousineau[25] that the exposure of normally fertilized eggs to deuterium depresses their RNA synthesis considerably. We thought it possible, therefore, that the protein synthesis evoked in unfertilized eggs during the period of deuterium storage might have been uncoupled from RNA synthesis.

The relevant data are plotted in *Figure 10*, and are taken from experiments run parallel to those represented by *Figure 9*. Here, the embryos were incubated during and after storage in D_2O (or in normal sea-water, for controls) with uridine-2-^{14}C, at an activity of 0·4 μc/ml and concentration of 3·3 μg/ml. An important technical point is that uridine is a precursor of both RNA and DNA in these cells, in consequence of which all preparations were digested sequentially with ribonuclease and deoxyribonuclease, with counting before, in between, and afterwards. Thus the counts incorporated into RNA alone could be obtained by difference.

Data points are given in the figure for the two-hour period of storage and for the first half-hour after removal of D_2O from the experimental suspensions. They are plotted as ratios of the counts in deuterated eggs to those in

control eggs. Remembering that the incorporation of uridine into controls, when unfertilized, is negligible (32 c/min/2 $\times$ 10^4 eggs at 120 minutes), we see that there has been no significant increase in the deuterated cells; on the contrary, since counts in the controls rise very slightly, probably again due to

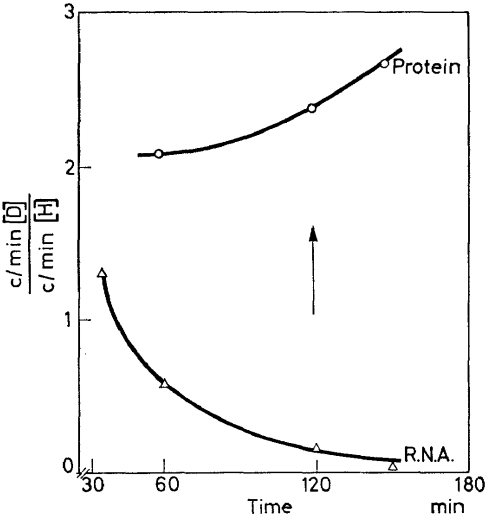

Figure 10. Ratio of counts incorporated into D_2O-treated eggs to counts in H_2O controls; precursor: uridine-2-^{14}C; counts for RNA'ase-digestible, acid-insoluble polymers (RNA); precursor: L-valine-1-^{14}C; counts in protein; eggs stored (unfertilized) in D_2O-sea-water or normal sea-water for 2 hours, then transferred to normal sea-water

oocyte contamination, there is produced a declining ratio in the plot. The controls make no protein, but the deuterated eggs do, both before and after washing; this yields the large and steadily rising counts ratio for protein.

Thus, deuterium parthogenesis effects the suspected uncoupling between protein and RNA synthesis in the unfertilized egg: the survival of protein synthesis for many hours after the D_2O has been removed indicates that this is not a trivial stimulation, but an authentic release of the normal pre-fertilization block. The question of interest is therefore the extent to which the mechanism of release by D_2O is related to that normally worked by fertilization.

DISCUSSION

The antimitotic action of heavy water is exerted, in higher cells, through an isotope mass effect upon the stability of cytoplasmic structures, notably the mitotic apparatus. The speed with which this effect is manifested, its relation to the exchange time between external water and cell water, and the observed physical properties of cytoplasm and its organelles before and after deuterium treatment, are all consistent with such a statement.

It is pertinent to inquire then what relation the observed inhibition of DNA synthesis bears to the antimitotic effect. Heavy water certainly stops

DNA replication in eggs[4], as well as in other higher cells. Bal and Gross[26], for example, have recently studied the kinetics of D_2O inhibition of cell division in root meristem, and here, the block comes at the end of the G_1 period of interphase, *i.e.*, just before DNA synthesis normally begins. The answer must be that, at least in the egg, cessation of DNA synthesis is a sufficient, but not a necessary condition for mitotic blockade, because deuteration imposes it even after DNA synthesis is finished in any cell cycle.

The inhibition of DNA synthesis is itself of interest in another connection, however, particularly because upon removal of D_2O, it is resumed at an unusually rapid rate, as though precursors for the terminal steps in replication (*i.e.*, copying of the primer DNA by the polymerase) had been piling up during the blockade. If this were a universal response of cells to D_2O, it might be assumed that the polymerase activity itself were being stopped by a large kinetic isotope effect; but this cannot be true, because the cells of micro-organisms can be adapted[27] to grow in pure D_2O, and hence replicate their DNA, and because other micro-organisms, for example, the fission yeast *Schizosaccharomyces pombe*, suffer an inhibition of cell division without a parallel depression of DNA synthesis[28].

The key will probably be found in differences in the organization of chromosomes, just as the differential sensitivity of the later stages of mitosis seems to reside in the presence or absence of a large achromatic apparatus. It is therefore possible that a fundamental addition to the structural complexity of chromosomes was made in the course of evolution; one candidate for material of this addition is protein. In view of recent evidence, it seems likely that when chromosomes become large, their structural complexity increases[24], as does the importance of proteins, probably histones, in that structure. The discontinuity in the antimitotic efficiency of deuterium against micro-organismal and higher cells suggests that the isotope effect is manifest only when relatively large structures are involved, larger than the single DNA molecule.

Indirect evidence for this point comes from the work of Crespi and Katz[30] and from that of Mahler, *et al.*[31], who showed that the thermal denaturation points of nucleic acids are insensitive to deuteration of the medium and even to complete deuteration of the molecule. Studies on proteins and polypeptides provide data which are difficult to apply to the problem of the isotope effect *in vivo*. Shifts in helix-random coil transition temperatures have been described by Calvin, *et al.*[32], and by Scheraga[33]. For ribonuclease in an aqueous solvent, deuteration of the medium produces a shift towards increased stability of the helical form, *i.e.*, such that intramolecular hydrogen bonds are favoured over bonds to solvent at any temperature. Studies on the screw parameters of fibrous helical synthetic polypeptides by Tomita, *et al.*[34], however, show that hydrogen bonds in the α-helix are somewhat lengthened upon deuteration. If any change in over-all stability of the structure were to result from deuteration, it would most likely be due to changed intrahelical van der Waals contracts. It is clear from all of these studies that in the present state of theory, only weak guesses can be made about the effect of deuteration on the stability of a given macromolecule in solution; much depends upon interactions with solvent and with other macromolecules.

The experiments with gelatin, despite the deficiencies of the model, therefore retain some relevance to the problem of the deuterium effect in living cells. They suggest that it is at least possible for systems actually or potentially crosslinked by hydrogen bonds to experience a large stabilization in D_2O.

We may finally take up, in this connection, the uncoupling of protein and RNA synthesis in deuterium parthenogenesis. The unfertilized eggs seem not to be wholly deficient in any of the components of a protein-synthesizing system: ribosomes, transfer RNA, amino-acids, energy co-factors, and activating enzymes (see, *e.g.*, ref. 20). There is some question about messenger RNA, which is the intermediary between structural genes and the synthesizing system, and which serves as a template for the sequential assembly of amino-acids. Fertilization switches on protein synthesis, and also, as has been shown recently[35, 36, 24], the synthesis of new RNA, probably messenger RNA. This suggests one type of control mechanism: absence of templates before fertilization and their synthesis at fertilization, resulting in the initiation of protein synthesis.

Some biological properties of the system cast doubt upon this attractive interpretation. The enucleate, artificially activated egg, for example, makes protein[22, 23]: since messenger RNA can only be made in the nucleus, this observation is puzzling, unless one assumes that cytoplasmic DNA, in an unknown state of polymerization and hence of unknown genetic competence, is the primer for post-fertilization messengers. Autoradiographic localization of radioactivity due to incorporated RNA precursors[25] does not support this hypothesis. The fertilized egg makes protein even when the synthesis of RNA has been shut down by treatment with the antibiotic actinomycin D[21, 24], and this is also in conflict with the idea that the templates for the first proteins made in development are synthesized after fertilization. The parthenogenesis data described above, demonstrating an effective uncoupling of protein and RNA synthesis, produce the same conflict. The most likely alternative explanation is that the unfertilized egg is pre-programmed to make certain proteins needed in early development; *i.e.*, the templates, in the form of messenger RNA, are built into the cytoplasm during oogenesis.

If this were so, then the regulatory mechanism would consist of an arrangement whereby the stored messages are inoperative until after fertilization. This could be accomplished in several ways: the messages might be sequestered in some cell compartment so as to prevent the attachment of ribosomes and the formation of polyribosomes, which are the sites of protein synthesis[37]. Alternatively, the polyribosomes might already be present, but non-functional, perhaps because of a stop placed at the end of each message, to prevent the release of ribosomes with finished polypeptide chains.

It is not yet possible to select among these, and other alternatives. But the release of the block to protein synthesis during a parthenogenetic treatment with deuterium can be exploited to obtain useful information. If the effect is of the same general nature as that operating on other structures, such as chromosomes and the mitotic apparatus, then it is possible that deuterium causes an enforced condensation between ribosomes and the stored messages. Such combination might normally be prevented by

unfavourable secondary structure in the messengers, or by a protein linker attached to each template. This would be changed at fertilization by a change in the ionic environment or by proteolytic activity. Both types of biochemical change in the cytoplasm are normal events of fertilization. It should be possible to examine these alternatives *in vitro*, using heavy water as a solvent for incorporation systems isolated from unfertilized eggs. Experiments of this sort, now in progress, will represent a novel application of the deuterium isotope mass effect.

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