

AN ELECTRON MICROSCOPE STUDY OF THE ENDOPLASMIC RETICULUM IN NEWT NOTOCHORD CELLS AFTER DISTURBANCE WITH ULTRASONIC TREATMENT AND SUBSEQUENT REGENERATION

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ABSTRACT

Ultrasonic treatment of the tails of *Triturus alpestris* tadpoles, at intensities of 8 to 15 watts/cm², at 1 megacycle/sec. for 5 minutes, disrupted the epidermis and caused pycnosis in individual cells of the muscle and neural tube, but caused no damage to the notochord that could be detected by light microscopy. Electron microscopy showed that this ultrasonic treatment disordered nearly all the endoplasmic reticulum (ER) of the notochord cells into irregularly rounded vesicles, but within 3 hours after treatment some parallel arrays of normal endoplasmic reticulum were seen near, and continuous with, the outer nuclear membrane. In addition, a re-ordering of the previously disordered ER took place throughout the cytoplasm, in some cases. A classification was made of the state of the ER as shown in electron micrographs of material fixed immediately, 3, and 24 hours after treatment. This showed that more than half the total endoplasmic reticulum in notochord cells was normal again by 24 hours after treatment.

INTRODUCTION

The ultrastructure of the cells of the urodele notochord in normal development has already been made the subject of an electron microscope investigation by Waddington and Perry (21). These authors refer to the endoplasmic reticulum (ER) as being ergastoplasm of the second type. It consists of serial arrays of flattened vesicles or cisternae, the opposite membranes of which are separated by about 1000 Å and may extend together for as much as 5 μ in the same direction. The cavity within the membranes contains material of a somewhat greater electron opacity than the cytoplasm outside. The ribosomes (about 150 Å in diameter) adhere to the outer surface of the membranes. Not all cell types contain endoplasmic reticulum, however, and all the ribosomes

of undifferentiated embryonic cells appear to be freely suspended in a cytoplasm without membranes.

The presence of endoplasmic reticulum is often associated with the ability of cells to synthesize proteins "for export," *e.g.*, in glands, as observed by Porter (16), Emmelot and Benedetti (4), Haguenu and Hollmann (8), and Jacob and Jurand (11). In the newt notochord at the stages selected for the present study, the endoplasmic reticulum is already well developed, but is still increasing in extent. Waddington and Perry (21) have suggested that ultrasonic vibrations might offer a convenient means of disrupting cytoplasm. Such a technique might permit the testing of ideas concerning the rate and con-

tol of protein synthesis by allowing a comparison to be made between normal cells and cells whose submicroscopic organisation has been partially disrupted. The present paper reports our first attempts to find such an experimental system.

Earlier studies by Selman (18), Selman and Counce (20), and Counce and Selman (2) have shown that it is possible to induce embryological and cytological abnormalities in certain living tissues by means of ultrasonic treatment. These abnormalities were studied with the light microscope only. All of them were induced by treatment with ultrasonic vibrations of a frequency of 1 Mcycle/sec. at intensities of 2.5 watts/cm² and less, using a generator identical in design to the one used in the present investigations. This apparatus and the calorimetric method for estimating ultrasonic intensities, which also were the same as those employed in the earlier studies, were described by Selman and Wilkins (19). At ultrasonic intensities greater than 2.5 watts/cm², the disruptive effects of cavitation usually make it impossible to work with living tissue after treatment, although the work done on plant seeds by Haskell and Selman (10) is an understandable exception.

PRELIMINARY EXPERIMENTS

Preliminary experiments were performed with a view to selecting the most appropriate conditions for the main experiment. Free-swimming larvae of *Triturus alpestris* were treated with ultrasonic vibrations at 1 Mcycle/sec. for periods of up to 5 minutes at intensities of 10 watts/cm². Although this is well above the cavitation level, the larvae were not all killed. After a recovery period, which varied from a few seconds to about 10 minutes, the surviving larvae showed swimming movements. The movements were usually abnormal, however, and the larvae examined under the binocular microscope exhibited damage to the tail surfaces, involving mainly the epidermis. A proportion of such treated larvae showed degeneration within 24 hours, especially after the longer treatments. Therefore, in subsequent experiments in which it was desired to study tail tissues of larvae after ultrasonic treatment, the tails were always excised in the region of the anus and cultured, if necessary, in Holtfreter's saline (one-tenth strength) with a phosphate buffer at pH 7.0. Such cultures are known to preserve the various tail tissues in normal condition without degeneration, so long as the regeneration area, which extends for about 1/2 millimeter from the excised surface, is avoided. Moreover, for culture periods up to 24 hours no strictly aseptic conditions are needed.

To allow a comparison to be made between treatments at different frequencies, some whole larvae were exposed to ultrasonic vibrations at 19 kilocycles/sec. The generator used was an M.S.E. Ultrasonic Disintegrator¹ which, according to the makers' pamphlet has a transducer output of 60 watts. Using the cylindrical transducer head of 3/4-inch diameter and tuning the generator to its fundamental frequency, we measured, by our admittedly slightly conservative calorimetric method, an ultrasonic output of 14 watts, which is equivalent to an average ultrasonic intensity of about 5 watts/cm². Under these conditions, vigorous cavitation was obvious. Larvae in a test-tube of water exposed to the output of this generator remained apparently undamaged so long as no part of them was at a pressure antinode, but one larva in a pressure antinode was seen to become totally homogenized within 2 seconds by the cavitation. Although no qualitative differences are known between the biological effects of ultrasonic treatment at different frequencies (see, for example, the work of Lepeschkin and Goldman, (14)), it would appear that, of the disruptive effects of cavitation which may be observed without optical aid, those which are produced at lower frequencies are usually the more violent. Apart from showing the node to antinode variation, which has been studied by Goldman and Lepeschkin (7), this particular generator also had the disadvantage of not being readily run at a lower output without detuning, hence all subsequent work was done with a megacycle generator whose output was more readily controllable. The cavitation damage produced by ultrasonic vibrations at 1 Mcycle/sec. tends to occur in randomly scattered patches of cells within a tissue, but no well marked node to antinode variation is evident in the ultrasonic effects produced by our apparatus. This is partly due to the lesser wave length, which is 1.5 mm in water at 1 Mcycle/sec. compared with 8 cm at 19 kilocycle/sec., and partly due to the different geometrical conditions which are less favourable to standing-waves in the generator at 1 Mcycle/sec.

Series of experiments were conducted in which the larvae were treated with ultrasonic vibrations at 1 Mcycle/sec., under conditions precisely the same as those in the main experiment, but, in this case, the whole larvae or their tail tips were prepared for examination under the light microscope. Two methods were used: in one, the tail tips were fixed in Mayer's acid haemalum; in the other, whole larvae were fixed in 5 per cent trichloroacetic acid with 1.37 per cent lanthanum acetate, stained with methyl green and pyronin, and subsequently serially sectioned transversely at 6 μ .

¹ Manufactured by Measuring and Scientific Equipment Ltd. of London.

The former method is suitable for the examination of epidermal tissues, but the latter is preferable for all other tissues. With these methods and fixing immediately after ultrasonic treatment, it became clear that treatment of larvae with intensities of between 8 and 15 watts/cm² for 5 minutes caused widespread disruption of the epidermis which was almost certainly due to cavitation. The deeper tissues suffered much less, and the notochord apparently not at all. When larvae were given the same treatment and the tail tips allowed to remain for up to 24 hours before fixation, the sectioned tail tips clearly illustrated the remarkable healing capacity of the epidermis. Even when the majority of the epidermal cells were destroyed and their structure disrupted, including the basal membranes and the tenuous mesenchyme beneath, within 3 hours the remaining epithelial cells would form, together with some cell debris in our case, a complete surface to surround the cylindrical central core of organs. This observation is in agreement with the work of Roguski (17) on amputated tails of *Xenopus* larvae. Three hours after treatment, and later, individual pycnotic nuclei in cells could be found scattered within the muscle tissue and the neural tube, but the general structure of these tissues was not destroyed. At no time was there any sign of cell or tissue damage to the notochord. The superficial epithelial tissues were thus most severely affected by the ultrasonic treatment, and there was a gradual decrease in damage towards the deeper tissues. The several possible reasons for this result will be discussed later.

No abnormal mitoses were observed at any time in the epithelial cells that had not been destroyed by treatment. Many normal mitoses were noted in that tissue, and these could be clearly seen by light microscopy in the whole-mount preparations. Sticky chromosome effects induced by ultrasonic treatment in other tissues have been observed previously by Selman (18).

MAIN EXPERIMENT USING ELECTRON MICROSCOPY

Larval stages of *Triturus alpestris* were selected between stages 34 and 41, according to the table of normal development constructed by Glaessner (5) for *Triturus vulgaris*. During these stages the forelimbs develop from a toe-less stub to a two-toe stage. The larvae, or their amputated tails, were subjected to ultrasonic treatment at a frequency of 1 Mcycle/sec. for 5 minutes at intensities of 8, 11, or 15 watts/cm² in an aqueous medium, pond water in the case of whole larvae, and Holtfreter's saline (one-tenth strength) in the case of the tails. The amputated tails were treated 5 at a time, and the treated group of tails was then divided into subgroups for immediate and later fixation. The treatment vessel was exactly as

described by Counce and Selman (2) and depicted in their text-figure 1, except that the aluminium ring was replaced by a polythene washer and the agar disc by a thin circular coverglass. The larvae were thus lightly held in the aqueous medium between parallel surfaces, the water being continually cooled by the cooling coil so that the temperature of the larvae could not rise more than 1 or 2 degrees above room temperature during the ultrasonic treatment. The fixative used was 1 per cent osmium tetroxide in sucrose solution with a Veronal-acetate buffer at pH 7.2, according to Caulfield (1) but with 3 per cent sucrose. After ultrasonic treatment, the tail tips were fixed for 15 minutes at 0°C, and then for 15 minutes at room temperature, or reincubated in Holtfreter's saline (one-tenth strength) until fixed, as before, at 3 and 24 hours after ultrasonic treatment. A substantial number of control larvae were similarly fixed. The fixed tails were dehydrated in a graded series of alcohols (35, 70, 95, and 100 per cent). After the absolute alcohol was replaced by two changes of epoxy-propane mixture (1:2) the tails were embedded in Araldite (6) and sectioned at about 600 Å with a Porter-Blum microtome. The sections were cut transversely, roughly midway between the excision surface at the anus and the tip of the tail, so that the region near the excision and the regeneration was not studied. The sections were mounted on grids coated with collodion-carbon films and then stained for increased contrast by floating on a solution of potassium permanganate (1 per cent) with uranyl acetate (2.5 per cent) for 20 minutes. The sections were examined and photographed using a Philips EM 75 or a Siemens Elmiskop I.

RESULTS

Since the appearance of the notochord cells was the same after ultrasonic treatment at 8, 11, and 15 watts/cm² for 5 minutes, the results will be combined. Electron microscopy clearly showed that the endoplasmic reticulum in the notochord after ultrasonic treatment was abnormal. The ribosomes were still bound to membranes, but the membranes assumed the general form of irregular rounded vesicles, as illustrated in Figs. 2 and 3, instead of showing the normal parallel arrangement illustrated in Fig. 1. Where the pairs of membranes did run together in the endoplasmic reticulum of the treated material, frequent in-pocketing and out-pocketing was seen which produced profiles resembling a series of inter-connecting bulbs.

The microstructure of the yolk platelets (13), mitochondria, pigment granules, nuclei, and notochord sheath appeared to be normal after

treatment. This was equally true whether the material was fixed immediately or at 3 or 24 hours after treatment. The treated cells exhibited no signs of degeneration. Occasionally, a few vacuoles about 0.5μ or less in diameter were found in the cytoplasm of cells after ultrasonic treatment, but these were not considered to be cavitation nuclei because they were not surrounded by zones of disordered ultrastructure. This was the only abnormality observed in treated notochord cells, apart from the abnormal ER.

The appearance of the endoplasmic reticulum in material fixed at 3 and 24 hours after treatment showed a trend towards normality. For instance, a substantial proportion of the photographed electron microscope fields (15 out of 65, if the data for fixations at 3 and 24 hours are combined) showed completely normal ER, with no abnormal forms, whereas material fixed immediately after treatment revealed no fields without some abnormal ER, and 70 out of 74 fields were all abnormal. Again, the proportion of electron micrographs which showed both normal and abnormal endoplasmic reticulum in different parts of the same field tended to be greater the later the time of fixation. In such cases, the normal ER, in parallel arrays, was nearly always adjacent to the nuclear membranes, whereas the abnormal ER was in the cytoplasm farther away from the nucleus. A typical case is shown in Fig. 4. Several instances were noted in which normal elements of the ER were continuous with the outer layer of the nuclear membrane, as described in the work of Waddington and Perry (21), Jurand (12), and other authors. This phenomenon was noted in material fixed at 3 and 24 hours after treatment, but not in material fixed immediately after treatment.

Our observations are consistent with the view that after the disorganisation caused by the ultrasonic treatment some new normal endoplasmic reticulum was formed at the nuclear

surface, commencing within 3 hours after treatment. Alternatively, the observations may be explained by assuming that a re-ordering process took place, beginning near the nuclear surface and spreading throughout the cytoplasm. In those cases where a return to the normal state was completed within 24 hours, the repair must certainly have been due to a re-ordering process for the most part, and not to the formation of new ER. The fact that regeneration and re-ordering of the ER can take place after ultrasonic treatment is, of course, further evidence against any reduction in cell viability having been induced in notochord tissue.

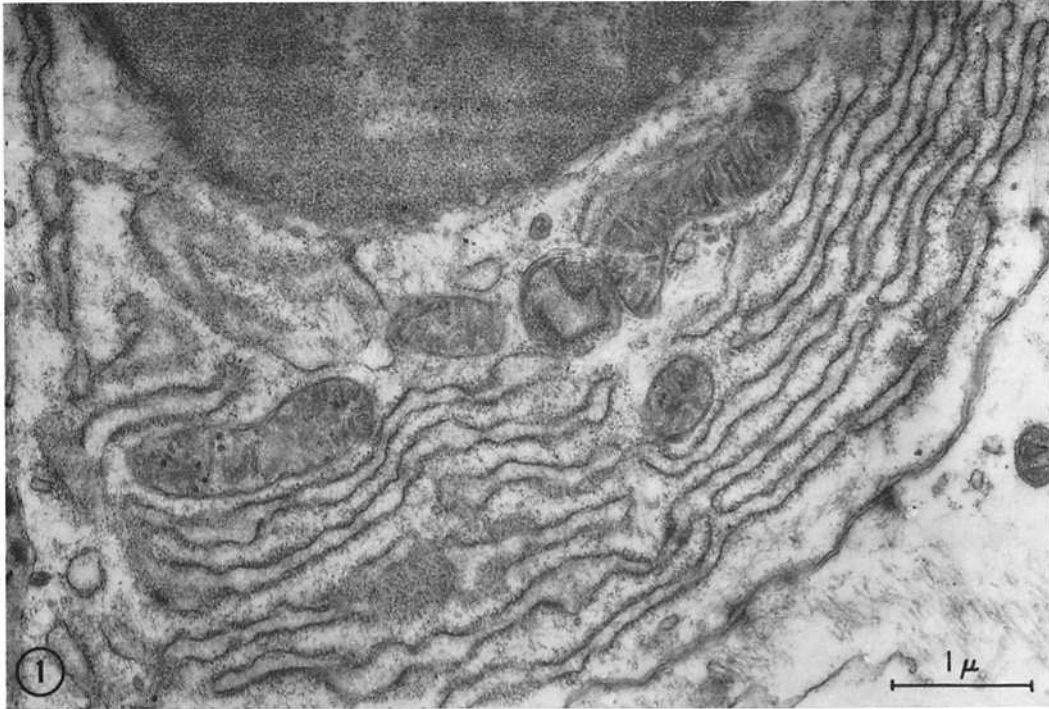
In the present investigation, we have tried to minimize the subjective element in assessing the significant features of the photographic records. No attempt was made to study the cytoplasm of cells on the fluorescent screen of the electron microscope. Instead, comparable fields showing cytoplasm were selected, usually by arranging to have a small portion of notochord sheath in one corner and a small portion of nucleus in the other. These fields were usually photographed at a magnification of 8,500. Enlargements were prepared and *all* the prints were examined for the appearance of the endoplasmic reticulum. The results are presented in the Table I. Occasionally, several photographs were taken of serial sections through the same cell. In such cases, all prints were used to assess the state of the ER, but the resulting assessment appears only as a single unit in Table I. Roughly equal proportions of prints derived from treatments at 8, 11, and 15 watts/cm² are found in each square of the last three rows of Table I, which confirms our previous assessment that each treatment produced a similar result.

An inspection of the results presented in Table I indicates that the ultrasonic treatment regularly produced a clearly recognizable immediate effect. Before treatment, 32 out of 34 cells had an endo-

The scale in each figure represents 1μ . All figures are of notochord cells from tadpoles between Glaessner stages 34 and 41. The photographs are electron micrographs of ultrathin transverse sections cut approximately midway between the anus and the tip of the tail.

FIGURE 1. Normal endoplasmic reticulum in control material not treated with ultrasonic vibrations. The ER extends in nearly parallel sheets. $\times 18,500$.

FIGURE 2. All the ER is in the form of abnormal, disordered, irregularly rounded vesicles. The material was fixed immediately after ultrasonic treatment at 11 watts/cm² for 5 minutes. The ribosomes still adhere to the membranous components of the ER. $\times 21,000$.



plasmic reticulum which was classified as entirely of normal form. Our judgment of what is normal is in agreement with the work of Waddington and Perry (21), and it should be understood that our criteria of normality would not necessarily apply to other tissues or other animals. After treatment, 70 out of 74 cells were entirely ab-

did not kill any of the notochord cells. A number of reasons are apparent as to why the deep tissues should have suffered less than the superficial ones. Since the damage to the epithelium, at least, is obviously cavitation damage, the explanation may be that cavitation occurred primarily in the water outside the tissue or at the tissue-to-water

TABLE I
Numerical Summary of the Results

	Time of fixation	Appearance of endoplasmic reticulum		
		Normal endoplasmic reticulum	Both normal and abnormal endoplasmic reticulum	Abnormal endoplasmic reticulum
	<i>hrs.</i>			
Control	—	32	2	0
Treated with ultrasonic vibrations	0	0	4	70
Treated with ultrasonic vibrations	3	5	9	16
Treated with ultrasonic vibrations	24	10	17	8

The table represents a classification of the observed states of the endoplasmic reticulum in newt notochord cells both in controls and in material treated with ultrasonic waves and then fixed at 0, 3, and 24 hours after treatment. In the three right columns are the numbers of notochord cells observed in photographs to have endoplasmic reticulum of the type indicated.

normal, and none was entirely normal. An inspection of the last three rows of the table illustrates a significant return to the normal form of ER which started before 3 hours after treatment and continued between 3 and 24 hours. More than half the ER was normal again by 20 hours after treatment.

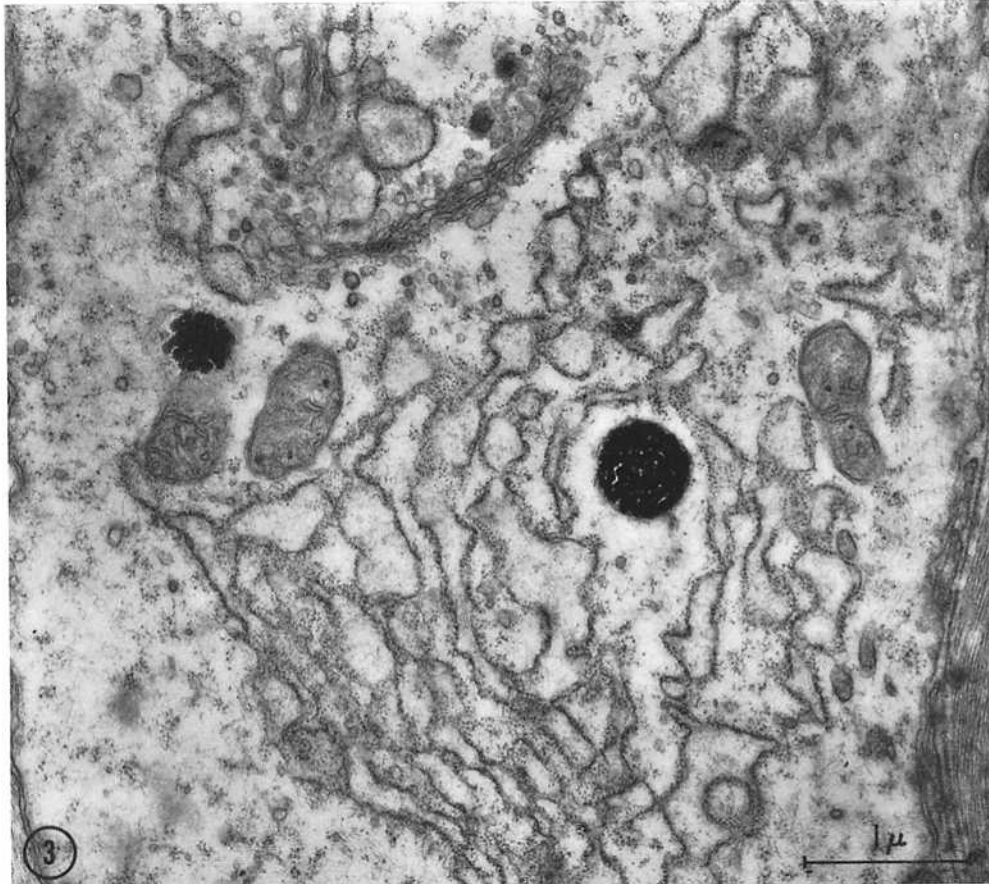
DISCUSSION

The preliminary experiments established that ultrasonic treatment destroyed a considerable part of the epithelial tissue and damaged individual cells in muscle and neural tissue, but apparently

boundary. This conforms with what is known about cavitation, which is favoured by surfaces and boundaries with water, and which has been shown by Goldman and Lepeschkin (7) to be inhibited by such media as agar gel. The effect of shock waves produced by collapsing cavities would presumably decline with, at least, the square of the distance from the point of collapse. Moreover, the ultrasonic intensity would diminish as the beam traversed the tissue, partly by acoustic absorption and partly by reflection at boundaries between media of differing acoustic resistivity. This might be the case at the edges of the noto-

FIGURE 3. All the ER is disordered. The membranes on either side of the vesicles show frequent inpocketing and outpocketing, giving profiles resembling a series of interconnecting bulbs. Notochordal sheath can be seen at the right. This photograph is of a tail treated with ultrasonic waves at 8 watts/cm² for 5 minutes and then fixed immediately. $\times 22,000$.

FIGURE 4. This photograph is of material treated at 11 watts/cm² for 5 minutes and then allowed to remain in Holtfreter's saline (one-tenth strength) for 3 hours before fixation. The ER is irregular and disordered except in an area of cytoplasm immediately outside the nucleus, in the upper half of the photograph, where it is in parallel sheets. Continuity exists between the ER and the outer nuclear membrane at the upper left corner of the photograph. The notochordal sheath can be seen at the opposite corner. $\times 22,500$.



chordal sheath, which would then play a protective role towards the notochord cells inside it. It is perhaps unlikely that this explanation is the complete one, but the effective ultrasonic intensity incident upon the notochord cells must be less than the intensities we have measured. On the other hand, it is just possible that certain types of differentiated tissues might be less sensitive to ultrasonic treatment than other, perhaps less differentiated, types when both are subjected to the same treatment.

Although we have made no direct microscope observations on notochord tissue while it was being treated with ultrasonic vibrations, it is probable that the effects on the endoplasmic reticulum which we have studied are caused by a general intracellular stirring-round which is induced by the ultrasonic waves but which has nothing to do with cavitation. In this case, the present observations are similar to those first made by Harvey and Loomis (9) on the chloroplasts of *Elodea* leaves, or by Selman and Counce (20) on the cytoplasm of *Drosophila* early-stage embryos, and it seems likely that all these cases should be discussed in terms of acoustic streaming, as in the work of Dyer and Nyborg (3) and Nyborg and Dyer (15). However, the whirling of chloroplasts in *Elodea* leaves and the stirring of yolky cytoplasm in *Drosophila* eggs are both induced by intensities much lower than those we have employed in this study.

In the present study, although the endoplasmic reticulum in the newt notochord was disordered by ultrasonic treatment, the ribosomes remained bound to their membranes. This is perhaps a disappointing result if, in future biochemical work, it is hoped to compare cells with ordered and disordered microstructure, because one of the main points of interest would be a comparison of the activities of bound and unbound ribosome systems in otherwise similar cells. Emmelot and Benedetti (4) showed that dimethylnitrosamine (DMNA) caused detachment of ribosomes from the membranes of the ER of rat liver, and that the same compound inhibited amino-acid incorporation. However, the possibility exists that

DMNA acts upon the ribosomal templates themselves, whereas the effects of ultrasonic treatment are more likely to be purely mechanical. The only conclusion that can be drawn at present is that the ribosomes are bound to the membranes by forces greater than those which normally maintain the membranes in parallel arrays. From the observation that a re-ordering takes place after the cessation of ultrasonic treatment, it may be concluded that the normal arrangement is the more stable, in which case the re-orientation may be envisaged as taking place through the agency of movements of the kind illustrated in many ciné-films of cytoplasm in living cells. Alternatively, it may be supposed that the endoplasmic reticulum is continuously ordered by a process quite unknown.

In the present work, the survival limit for notochord tissue treated with ultrasonic waves was not reached, so the possibility remains that further studies with greater ultrasonic intensities, perhaps under slightly different experimental conditions, might produce a more drastic disturbance of the endoplasmic reticulum, or even qualitatively different effects, while still allowing the tissue to survive. The drastic disruption of the epithelial tissues that was observed, however, should probably be taken as indicating that the survival limit for notochordal tissue, although not reached, was, in fact, not very much greater than that reached under the conditions of the present experiments.

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