Because the centrally acting barbiturates produced an effect in the dogfish and because another centrally acting compound, M-99, had no effect in the dogfish (MDIBL Bulletin 8:6, 1968) we studied the effects of morphine on the overt behavior and respiration of the dogfish (measured by observation of gilling). Doses up to 20 mg/kg i.a. had no effect on the overt behavior or gilling of the dogfish when observed for 6 hours post-injection. This is in sharp contrast to the effects of the drug in mammals where morphine is a primary and continuous depressant of respiration, usually occurring maximally within 7 minutes after i.v. administration.

1969 #4

RELATIONSHIP OF MACROMOLECULAR SYNTHESIS TO MORPHOGENESIS IN Fundulus heteroclitus EMBRYOS

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The results of previous studies led to the tentative conclusion that temporal control over protein synthesis during the first few minutes after fertilization is especially critical for normal development of the teleost Fundulus heteroclitus (Bull. MDIBL 8:13-15, 1968). The investigations reported here confirm and extend this observation.

Pactamycin (20 μg/ml) has been shown to be an effective agent for inhibiting protein synthesis in Fundulus embryos. However, this had not been clearly demonstrated during the immediate post-fertilization period. Experiments were conducted to determine the extent of inhibition of protein synthesis by pactamycin when the inhibitor was added at time periods soon after fertilization (15 seconds, 2, 5, 30, and 60 minutes). Protein synthesis was measured as the extent of incorporation of ^{14}C-labeled amino acids (valine or a mixture) into hot trichloroacetic acid insoluble proteins over a two hour incubation period. Although incorporation rate is very low during the immediate post-fertilization period, it could be determined from the data that pactamycin inhibited about 70%. The 30% of protein synthesis allowed may represent a fraction which is not affected by pactamycin. However, it also may represent a differential rate of penetration of the embryo by the amino acid and the inhibitor. At least it now seems clear that protein synthesis is severely diminished by pactamycin at all stages of Fundulus development.

The ability of early embryos to undergo cell division while incubating in pactamycin was studied. In one series of investigations, several experiments were conducted in which the embryos were placed in pactamycin at various time intervals following fertilization and kept in the inhibitor continuously until termination of the study. Under normal conditions the embryos undergo first cleavage about two hours after fertilization. If the embryos were in pactamycin during the first 30 seconds post-fertilization, no cleavage occurred. When incubated in pactamycin starting any time between 30 seconds and 3 minutes post-fertilization, one cleavage occurred, the number of embryos dividing increasing as the time from fertilization increased. Introducing the inhibitor 3 minutes post-fertilization or later allowed 2 cleavages to occur and if the embryos were spared for 5 minutes or more 3 cleavages occurred. In all cases the cell division lags considerably behind the controls, the embryos are abnormal, and within two days all are dead. What appears to be particularly significant is that during the first ten minutes following fertilization, syntheses, which can be inhibited by pactamycin, are occurring which are

required for three subsequent series of cell division. Thus it might be considered that preparation for the first several series of cell divisions in Fundulus is dependent on protein synthesis which occurs under rather rigid temporal control during a short time period after fertilization. It is also of interest to note that this preparation for subsequent cleavages occurs prior to the first cleavage.

Another series of experiments was conducted to determine the relation of pulse time and pulse length of pactamycin to cleavage and normal development. This was of interest not only because of the effects shown on cleavage but also because earlier studies had shown the effect of pactamycin on protein synthesis to be reversible. The pulse times used were 0, 1/4, 1, 2, 3, 4, 5, 10, 30, and 60 minutes post-fertilization, the time at which incubation of the embryos in pactamycin was begun. Pulse lengths for each of these pulse times were 15, 30, 60, and 120 minutes, and one day. The development of these embryos was observed for 30 days. The extent of development, of differentiation whether normal or abnormal, was clearly directly related to pulse length and to the amount of time after fertilization the pulse was begun. A consistent pattern was observed: those embryos placed in pactamycin early or kept in for the longer periods showed cleavage (abnormal) to approximately 8 cells, followed by arrested development and death; embryos placed in the inhibitor 2 minutes post-fertilization and then removed to normal medium 15 or 30 minutes later showed a few cases of keel formation (elongate cellular masses with pigment); similarly, if incubation in pactamycin was delayed until 3, 4, and 5 minutes post-fertilization, increasing numbers of the keel-like structures developed; when the inhibitor was added 10 minutes post-fertilization, definite signs of cephalization and recognizable body form were evident in the keels when the pulse length was 30 minutes or less; those incubations in which the inhibitor pulse began 30 and 60 minutes post-fertilization showed many embryos which developed normally when the pulse length was 30 minutes or less.

In all cases the effect of the inhibitor was evident. Even where the embryos appeared to be normal after 30 days they lagged behind the controls in rate of development. Thus it appears that at least during the first 60 minutes of development there are periods of protein synthesis which must occur at their appointed times, in an ordered sequence, to insure normal development. These periods are in the first few minutes after fertilization. Later periods of protein synthesis may be delayed without seriously affecting normal development other than rate. Recovery from this inhibited protein synthesis cannot occur if the inhibition lasts much longer than 30 minutes. Thus we conclude that previsely controlled protein syntheses during the immediate post-fertilization period are required for normal cleavage and subsequent events of differentiation and morphogenesis.

It had been previously reported that late stage Fundulus embryos incubated for two hours in Actinomycin D were able to fully recover their ability to incorporate uracil into RNA after removal of the inhibitor (Bull. MDIBL 8:13-15, 1968). This seemed consistent with the finding that such a pulse with Actinomycin D had no effect on subsequent morphogenesis. However, if embryos are incubated in this inhibitor (20 μg/ml) during cleavage stages, especially within the first two hours post-fertilization, profound effects on subsequent morphogenesis are observed (Exptl. Cell Res. 44:471-488, 1966). Therefore, recovery of RNA synthesis ability was examined in early embryos following a two hour pulse of Actinomycin D. It was found that after approximately one day following relief from the inhibitor normal rates of uracil incorporation into embryo RNA were achieved. Thus, although the effects of Actinomycin D, administered soon after

fertilization, on morphogenesis are irreversible, the total synthetic rate of RNA, even in anomalous embryos, may be restored. This further substantiates the hypothesis that a precise sequence of RNA syntheses is required during early stages for successful morphogenesis.

The relatively long life of certain messenger RNA molecules during oogenesis and embryonic cleavage stages appears to be well established. A number of explanations for the stability of these mRNAs may be offered, including binding with a protein, sequestering from cellular ribonuclease, or the absence of ribonuclease during early stages of embryogenesis. In order to narrow the field of possible explanations, the latter hypothesis was tested, i.e., the presence of ribonuclease was tested for at all stages of Fundulus development. The assay was a modification of that by Zimmerman and Sandeen (Anal. Biochem. 10:444-49, 1965). The enzyme was incubated with polycytidylic acid and the extent of release of $HClO_4$-soluble nucleotides was measured by observing the absorbance at 268 mμ. The embryo extract assayed was the 100,000 x g supernatant obtained from centrifuging an homogenate prepared in 0.005 M Tris buffer, pH 8.2. The results of these assays showed measurable quantities of ribonuclease present in all stages of Fundulus embryos, remaining at about the same level throughout development. Thus, the absence of ribonuclease from cleavage stages of Fundulus embryos would not serve as a reasonable explanation for the stability of mRNA.

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1969 #5

BRAIN BARRIER SYSTEMS: AGE AND SPECIES DIFFERENCES

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We have examined brain barrier systems in several species of Chondrichthyes and one species of Agnatha using ^{14}C-inulin. Results indicate differences both between species and between age groups of a single species.

Plasma ^{14}C-inulin concentration was maintained relatively constant in Chondrichthyes by repeated i.m. injections: Myxine received a single injection. Chromatographs of tissue samples showed that ^{14}C-inulin accounted for more than 90% of tissue radioactivity in plasma of Myxine and in plasma, CSF and brain of Squalus acanthias (mature and pups). Potassium concentration of Myxine plasma was higher than reported in the literature (12.2 versus 9.6 meq/kg H_2O), raising questions concerning the physiological condition of these specimens.

Distribution ratios for ^{14}C-inulin between brain and plasma (R_{Br}) and between CSF and plasma (R_{CSF}) are summarized in Table 1. Values for rats are included for comparison (J. Physiol. 169:816-50, 1963; Arch. Neurology 12:284-95, 1965). R_{Br} and R_{CSF} in mature dogfish (S. acanthias), nurse shark (G. cirratum), and hagfish (M. glutinosa) are higher than in adult rats. Increased inulin content of brain is not a feature common to all lower vertebrates, however, as shown by values for rays (D. sabina and D. sayi), significantly more inulin was found in CSF and brain of immature than adult dogfish suggesting that in this species, as in mammals, the ability to exclude substances from the central nervous system develops with age. This conclusion was further supported by comparisons of CSF and plasma protein concentrations (Lowry)