The Effects of 2-Acetylaminofluorene on the Embryonic Development of the Zebrafish

II. Histochemical Studies*

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Although some studies have been made on the effects of carcinogens on the embryonic development of various animals (1, 5, 9, 15, 17, 18, 22), little effort has been expended in studying the mode of action of these chemical compounds. A previous paper (11) described the teratogenic effects of 2-acetylaminofluorene (AAF) on the development of the zebrafish; the abnormalities included a distortion of the embryonic axis; cellular degeneration of the liver; disorganization of the brain, spinal cord, and sense organs; and epidermal hyperplasia.

Cancerous growths may be related to an interference in protein synthesis (6-8, 13, 14, 16-21, 29, 30), and, in view of the fact that abnormal growth seems to be an expression of abnormalities in the chemical make-up of cells (29), histochemical methods were employed in this investigation to determine whether the anomalies induced by AAF would be reflected in changes of the ribonucleic acid levels of various organs. If RNA decreases, then an interference in protein synthesis may be indicated (4). Furthermore, since the carbohydrates appear to be very important in morphogenesis, at least in the chick embryo (25-27), it was decided to determine the effect of AAF on the glycogen levels of the zebrafish embryos. In essence, then, the purpose of this phase of the investigation was to determine whether AAF affects the metabolism of nucleic acid and glycogen in the developing zebrafish embryo.

MATERIALS AND METHODS

Abnormal embryos resulting from AAF action were obtained primarily from the experiments described in a preceding paper (11). These experiments were terminated when all the control embryos hatched. Prior to preservation, the unhatched experimental embryos were removed from the chorion by means of dissecting needles. Both the control and experimental embryos from each lot were preserved in approximately equal numbers in (a) Bouin's solution for histological study, (b) cold Carnoy's fixative for RNA determinations, and (c) cold picric-alcohol-formal solution for glycogen determinations. At least six to eight embryos from each experimental lot were serially sectioned and stained for each histochemical method.

Ribonucleic acid.—Following dehydration, embedding, and sectioning at 8 μ, the tissues were stained with buffered methylene blue (29), and the demonstration of ribonucleic acid was accomplished by comparing the distribution of stainable material in parallel digested and undigested sections. The digestion of these sections was carried out in 5 per cent perchloric acid (as well as ribonuclease—1 mg/ml distilled water) (29), the tissues being incubated for 2 hours at 37° C. Experimental slides were always compared with serially sectioned control embryos (reared in water) from each experiment, stained in the same way.

Glycogen.—Following fixation in cold picro-alcohol-formal, dehydration, embedding, and sectioning at 8 μ, the tissues were stained with Best's carmine. The controls were digested in saliva for 80 minutes at 37° C.

RESULTS

Ribonucleic acid.—Although embryos ranging from early cleavage to larval stages were exposed to AAF, the effect on ribonucleic acid levels of the embryos was essentially the same. There was a slight difference when higher concentrations of AAF (0.03 per cent) were used, but only to the extent that there was a greater reduction in the RNA contents of organs. In embryos treated with AAF, the organs which showed significant decreases in RNA were the following: the liver, brain, spinal cord, optic cup, auditory vesicles, the gastric and intestinal mucosa. The liver exhibited the greatest decrease in ribonucleic acid, and the cells which were undergoing degeneration contained only traces of RNA. The gastric and intestinal mucosa exhibited the slightest decrease in RNA content. The RNA levels of the remainder of the organs were unaffected by AAF, and the hyperplastic epidermal cells also showed no changes in RNA.
When embryos were exposed for short periods to AAF and then transferred to water, the affected organs, namely, the central nervous system, the sense organs, and the liver, regained the normal levels of RNA parallel to a morphological recovery. This was confirmed by histological examination of serial sections. To demonstrate the ability of the embryos to recover from the effects of AAF, Table 1 is presented. With reference to the liver, the data indicate that the recovery from the effects of the carcinogen was dependent upon the concentration and duration of exposure to AAF. Table 1 indicates for embryos exposed to AAF

**TABLE 1**

**EFFECT OF AAF ON LIVER RNA OF ZEBRAFISH EMBRYOS**

Intensity is graded from 1+ (weakest) to 4+ (strongest). Data apply to embryos of 4 days; initial stage exposed: mid-gastrula (i.e., epiboly).

<table>
<thead>
<tr>
<th>CONCENTRATION</th>
<th>DURATION OF EXPOSURE (HRS.)</th>
<th>PROLONGED EXPOSURE TO AAF</th>
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</thead>
<tbody>
<tr>
<td>AAF</td>
<td>AAF PRIOR TO TRANSFER TO WATER</td>
<td>LIVER RNA</td>
</tr>
<tr>
<td>0.002</td>
<td>4+</td>
<td>4+</td>
</tr>
<tr>
<td>0.005</td>
<td>4+</td>
<td>4+</td>
</tr>
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</table>
| 0.030 | 4+ | 4+ | 2+ | 2+ | 2+

NOTE:Liver of control embryo (age, 4 days) shows an intensity of 4+.

from the gastrula stage that: (a) exposures up to 8 hours in 0.002 per cent AAF caused no disturbance in the RNA content of the liver. If exposures were extended to 16 hours or more, normal RNA levels were not regained by the liver. (b) Embryos may be exposed up to 8 hours in 0.05 per cent AAF without causing a disturbance in the RNA level of the liver. However, exposures of 8 hours or more did not allow the return of RNA to its normal level in the liver. (c) Exposures of 4 hours or more to 0.03 per cent AAF caused a significant decrease in the RNA content of the liver, and the recovery to normal levels did not occur on transfer of the embryos to water.

The brain, optic cups, and spinal cord recovered from the effects of AAF in a similar manner.

**Glycogen.**—In the control embryos of the zebrafish at hatching, several organs contained an appreciable amount of glycogen. The organs in order of the greatest amount of glycogen were: the liver and yolk-sac syncytium (parablast), somatic musculature, heart and yolk (traces). There was no appreciable amount of glycogen in the brain (Fig. 1), spinal cord, eyes, notochord, epidermis, gut, pronephric tubules, and air bladder.

The liver of the zebrafish is quite a large organ at the time of hatching. It appears as a triangular structure on the ventro-lateral surface of the gut and is dorso-lateral to the yolk mass. The cytoplasm of the liver cells and the yolk-sac syncytium contain a heavy deposit of glycogen (Fig. 3). On the other hand, the muscle fibers of the somatic musculature contain a moderate amount of glycogen.

AAF primarily affected the glycogen content of the liver and the yolk-sac syncytium (Fig. 4). When embryos were exposed to high concentrations of AAF, the liver and yolk-sac syncytium were completely depleted of glycogen. At the same time, deposits of glycogen were noted in the brain (Fig. 2) and in the spinal cord. Accumulations of glycogen also occurred in the optic cups and the otic vesicles. The glycogen content of the somatic musculature remained relatively constant. The effect on the glycogen levels of the organs of the zebrafish embryo was essentially the same regardless of the stage of development exposed to AAF.

To illustrate the effect of AAF on the liver glycogen of the zebrafish embryo, Table 2 is presented.

**TABLE 2**

**EFFECT OF AAF ON THE GLYCOGEN CONTENT OF THE LIVER OF THE ZEBRAFISH EMBRYO**

Intensity is graded from 1+ (weakest) to 4+ (strongest); 0 indicates absence of glycogen. Data apply to embryos of 4 days. Initial stage exposed: mid-gastrula (i.e., epiboly).

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<td>0.030</td>
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NOTE: Liver of control embryo (age, 4 days) shows an intensity of 4+.

The results show that a return of liver glycogen to normal levels was dependent upon the length of exposure to AAF and the concentration of AAF. For example, if an embryo was exposed to 0.005 per cent AAF for 16 hours or more, glycogen was not restored to the liver. However, if the exposure to this concentration of AAF was reduced to 4 or 8 hours, some glycogen was regained by the liver. For concentrations of 0.002 per cent AAF, exposures up to 16 hours would allow the liver to retain normal glycogen levels, but exposures of 32 hours or more caused a definite decrease in glycogen.

**DISCUSSION**

The results of this investigation show that definite changes in the concentration of RNA and glycogen accompany the cellular disorganization in abnormal embryos induced by AAF.

In embryos treated with AAF, there was a consistent decrease of the RNA content of the liver,
nervous tissue, sense organs, and the mucosal lining of the digestive tract. Of these organs, the liver exhibited the greatest reduction of RNA. The studies of Brachet (2, 3) and Hisaoka and Hopper (11, 12), which utilized metabolic inhibitors, indicate that a specific interference in RNA may contribute to abnormal development in amphibian and teleost embryos, respectively. The results of the present study also seem to indicate that the reduction of RNA levels caused by AAF is in some way associated with the induction of anomalies in the zebrafish embryos.

The teratogenic effects of AAF on the zebrafish embryo are also correlated with an abnormal carbohydrate metabolism. In fact, the action of AAF in depleting the glycogen content of the embryonic liver seems much more pronounced than parallel RNA decreases. In most instances the liver and yolk-sac syncytium were completely lacking in glycogen stores, while the brain, spinal cord, optic cups, and auditory vesicles contained an abnormal deposit of glycogen. The deposition of glycogen in these organs cannot be explained at the present time, but these deposits appear to be correlated with a corresponding loss of glycogen from the liver. In the zebrafish embryo, the ventral border of the liver is in direct contact with the yolk mass. Together with the yolk-sac syncytium, the liver seems to be involved in the absorption of nutriments from the yolk mass, and it is therefore possible that the impaired function of these structures might enhance the formation of anomalies by preventing the transport of glucose by means of the circulatory system to such organs as the brain and spinal cord. The transport of glucose would be further hindered in those embryos in which AAF caused the cessation of circulation.

The results of this investigation seem to support other evidence that carbohydrates are an important requirement for normal developmental processes. Spratt (28) found that the maximal degree of histogenesis in the brain and optic cups of chick embryos occurred only in the presence of an adequate amount of glucose, and Pomerat and Wellmer (24) reported that the growth of tissues in vitro is also associated with the direct breakdown of glucose.

Experiments in which zebrafish embryos were exposed to AAF for short periods of time demonstrate that a complete recovery from the teratogenic effects of AAF is correlated with the return to normal levels of RNA and glycogen. Although definite conclusions as to the mode of action of AAF cannot be drawn from the results of this investigation, there is a suggestion that RNA and glycogen levels are important factors for the normal differentiation and morphogenesis of the zebrafish embryo.

SUMMARY

1. The mode of action of 2-acetylaminofluorene (AAF) in inducing the anomalous development of the zebrafish embryo was studied by histochemical methods. Glycogen (Best’s carmine technic) and ribonucleic acid (methylene blue technic) levels were determined in serially sectioned embryos.

2. The ribonucleic acid content of the liver, brain, spinal cord, sense organs, and mucosal lining of the digestive tract decreased in embryos which were exposed to AAF.

3. Carbohydrate metabolism was greatly impaired, as evidenced by the deposition of glycogen in the brain, spinal cord, and sense organs of the embryo. Also, AAF caused a decrease or depletion of liver glycogen. The liver of the zebrafish embryo appears to be the primary target organ of AAF.

4. The complete morphological recovery of an embryo from the teratogenic effects of AAF, when the embryo was returned to fresh water, was correlated with a return to normal levels of RNA and glycogen. There was a suggestion that RNA and glycogen are important factors in the processes of differentiation and morphogenesis in the zebrafish embryo.

ACKNOWLEDGMENTS

Grateful acknowledgment is made to Dr. Arthur F. Hopper and Dr. James H. Leathem for their advice and encouragement during the course of this investigation.

REFERENCES


Figs. 1-4.—Photomicrographs of sections (8 μ) through embryos of Brachydanio rerio. The sections were stained with Best’s carmine.

Fig. 1.—Section through the brain at the level of the eyes of a control embryo of 4 days at 26° C. X140.

Fig. 2.—Section through the eyes and brain of a 4-day embryo following continuous exposure to 0.002 per cent AAF from early cleavage. Loose cells in the ventricles contain glycogen. X140.

Fig. 3.—Section through the control embryo of 4 days at the level of the pectoral fins. Note glycogen in the liver, yolk-sac syncytium, and somatic musculature. Li, Liver; YS, Yolk-sac syncytium. X140.

Fig. 4.—Section through the level of the pectoral fins of a 4-day embryo following continuous exposure to 0.002 per cent AAF from early cleavage. Liver possesses only traces of glycogen. X140.


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