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# ACTIVITY OF CHOLINESTERASES IN THE JAPANESE QUAIL EMBRYO

## EFFECTS OF DICHLORPHOS ON THE EMBRYONIC DEVELOPMENT

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**Abstract**—Cholinesterase activity is detectable in the Japanese quail embryo, in the yolk and subembryonic liquid, but not in the albumen. Obviously, this enzyme is deposited by the hen into the yolk and from there it is transferred to the subembryonic liquid. In contrast, in the embryo the enzyme is synthesized by itself and the amount increases with the age of the embryo. By using BW284c51 1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one bromide and ISO-OMPA tetraisoprophylpyrophosphoramide as inhibitors, it was found that the enzyme in the embryo is predominantly acetylcholinesterase (EC 3.1.1.7), whereas that in the yolk and subembryonic liquid is butyrylcholinesterase (EC 3.1.1.8). Both types are inhibited by dichlorphos. However, the embryonic enzyme activity is restored within 8 hr, whereas that in the subembryonic liquid remained inactive at least for 72 hr after inhibition. Enzyme inhibition leads to retardation of the development, to reduced accumulation of glucose and amino acids in the subembryonic liquid and finally to death of the embryo, suggesting that the developmental retardation is due to the restricted supply of glucose and amino acids. Surprisingly, most of the embryos die when the embryonic enzyme activity has again been restored.

Cholinesterases are enzymes which hydrolyse cholinesters faster than other esters. They can be divided into two groups known as acetylcholinesterase (AChE†; EC 3.1.1.7) and pseudocholinesterase or butyrylcholinesterase (BuChE; EC 3.1.1.8). The criteria on which this division is based include differences in substrate and inhibitor specificities and in kinetic constants.

Concerning the function of these two types of enzymes, there is no doubt that inactivation of acetylcholine is the major function of the AChE in neurotransmission; however, several features of AChE suggest that it may have other functions [1]. As to BuChE no definite functions have been established so far.

It has been speculated that both AChE and BuChE mediate embryonic processes. On the basis of earlier results obtained from embryonic chicks, cholinesterases seem to display a morphogenic capacity [1–3] and blockage of the enzymatic activity is followed by teratogenic effects [4–6].

In order to elucidate the function of cholinesterases in avian embryos it seems reasonable to follow the distribution of enzyme activity in the various egg compartments. Therefore, we measured cholinesterase activity in the embryo and the extraembryonic compartments and followed the effect of dichlorphos, a potent inhibitor of AChE and BuChE, on metabolic and developmental parameters.

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† Abbreviations: AChE, acetylcholinesterase (EC 3.1.1.7); BuChE, butyryl- or pseudocholinesterase (EC 3.1.1.8); BW284c51, 1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one bromide; ISO-OMPA, tetraisoprophylpyrophosphoramide.

### MATERIALS AND METHODS

*Chemicals*. Chemicals were obtained as follows: enzymes and cholinesterase assay kit from Boehringer Mannheim, dichlorphos from Labor Dr. Ehrensdorfer, Augsburg, BW284c51 (1,5-bis-(4-allyldimethylammoniumphenyl)pentan-3-one bromide) and ISO-OMPA (tetraisoprophylpyrophosphoramide) from Sigma (Munich), dextran sulphate from Serva (Heidelberg). All the other chemicals were from Merck (Darmstadt).

*Japanese quail embryos*. The experiments were performed with Japanese quail eggs from our own breed. We used a system of *in vitro* culture because of its advantages compared to the *in ovo* system, i.e. exact administration of agents into a defined compartment, continuous observation of development and rapid detection of anomaly. The embryos were cultured according to the method described by Wittmann *et al.* [7]. Cultivation was started after incubation of the intact eggs for 2 days. In this way malformations, which occur when the eggs are cultured from the very beginning of incubation, could be prevented. The developmental stages were recorded according to Hamburger and Hamilton [8], as modified by Padgett and Ivey [9].

*Cholinesterase activity*. The embryos were weighed and homogenized in a Potter–Elvehjem homogenizer after adding a 4-fold amount of aqua dest. The homogenate was centrifuged (5 min, 14,000 *g*) and the supernatant was used for measuring the enzymatic activity. No cholinesterase activity was detectable in

the precipitate of yolk and subembryonic liquid. The precipitate of the embryo contained about 10% of the total cholinesterase, which was neglected in the data. The egg albumen was treated in the same way. In the case of the subembryonic liquid the sample was centrifuged (5 min, 14,000 *g*) without homogenization. Before measuring cholinesterase activity in the yolk, lipids were removed by means of dextran sulphate [10].

The enzymatic activity was assayed according to Ellman *et al.* [11], using acetylthiocholine as a substrate. Thiocholine formed by the enzyme at 25° reacts with dithiobisnitrobenzoic acid to thionitrobenzoic acid, which was recorded automatically each minute at 405 nm. A microplate assay was used for measurement. Acetylthiocholinejodide (156 mM) and dithiobisnitrobenzoate (0.25 mM) were dissolved in phosphate buffer (52 mM, pH 7.2). To a 20 µL sample 6.7 µL acetylthiocholinejodide solution and 200 µL dithiobisnitrobenzoate solution were added.

The total activity in the embryo and in the yolk was calculated on the weight basis. The weight of the yolk was recorded after cooking the egg and separating the yolk from the other compartments. The activity in the subembryonic liquid was assayed on the basis of its total volume, which was measured by means of the dilution of methyl orange. For this purpose 100 µL of a 0.03% methyl orange solution were injected into the subembryonic compartment and the egg was carefully shaken in order to get a homogenous solution. Ten minutes later two samples (100 µL) from different parts of the subembryonic liquid were drawn, the protein of which was precipitated by adding 1.3 mL of 0.33 M $HClO_4$, and centrifuged (1000 *g*, 10 min). To remove the lipids 5 mL $HCCl_3$ were added and the solution was vigorously shaken for 30 min. The two phases were separated by centrifuging (3000 *g*, 10 min). The extinction of the coloured phase was recorded at 496 nm and the volume calculated by means of a standard curve.

*Characterization of the cholinesterases.* The enzymes were characterized by using BW284c51, a known potent inhibitor of AChE and by ISO-OMPA, which inhibits mainly BuChE. In preliminary experiments the inhibitory capacity of BW284c51 and ISO-OMPA was determined by means of commercially available preparations of the enzymes (Sigma). A concentration of $10^{-4}$ mol/L BW284c51 inhibited isolated AChE by 94% whereas no effect was observed with isolated BuChE. On the other hand, the same concentration of ISO-OMPA exerted almost no effect on AChE, but inhibited 95% BuChE.

*Assessment of the survival rate.* Embryos were observed twice a day and considered to be dead when there was no circulation in the blood vessels of the yolk sac or the allantois. The percentage of surviving embryos was calculated on each day of incubation.

*Metabolites.* In order to assay the amino acids the subembryonic liquid was deproteinized by 3.0% (v/v) trichloroacetic acid. After centrifugation ether was added to the supernatant, the solution was mixed thoroughly and then the organic phase which contained the $CCl_3COOH$ was removed. This procedure was repeated until pH 7 was attained in the hydrophilic phase. The amino acids in the hydrophilic phase were transformed to the *o*-phthalaldehyde derivatives, which were separated and recorded by fluorescence in a HPLC apparatus as described by Cronin and Hare [12]. For the separation of the *o*-phthalaldehyde derivatives the HPLC column (GROH-Amino-OPA 3 µm; 150 mm × 4.6 J.D., Fa. Gynkotek, Munich) was equilibrated with a buffer (A) containing 6.0 g sodium acetate, 3.1 g bromic acid and 100 mL methanol (pH 6.0). After sample injection (20 µL) a stepwise elution with methanol (B) was performed: 0–18 min the gradient was run up to 17% B, 18–24 min to 28%, 24–28 min to 39%, 28–42 min to 70%. Flow rate was 1.0 mL/min, excitation wavelength 355 nm, emission wavelength 450 nm.

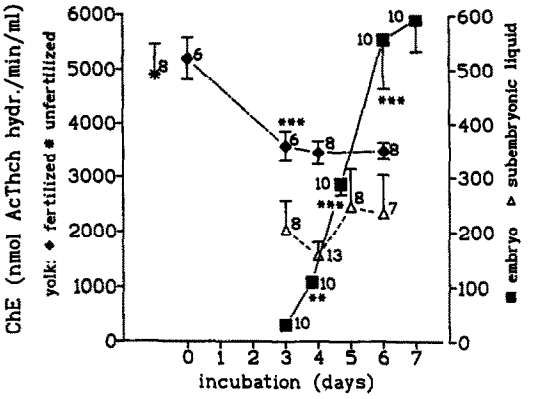


Fig. 1. Total cholinesterase (ChE) activity in the subembryonic liquid, yolk and embryo; **P < 0.01, ***P < 0.001 compared with the data of the preceding day. AcThch hydr. = acetylthiocholinejodide hydrolysed.

In order to determine glucose, the subembryonic liquid was deproteinized with 0.6 M $HClO_4$. After centrifuging (10 min, 14,000 *g*) the supernatant was neutralized with $KHCO_3$ and an aliquot used to determine glucose according to the method of Bergmeyer [13].

*Statistics.* The data are expressed as means ± SD. The significance of the differences between the means was determined by the Student's *t*-test. Differences of the survival rate, however, were statistically evaluated by means of the chi-quadrat test. In the figures the sample size is given near the symbols or in parentheses in the legend.

## RESULTS

As Fig. 1 shows, acetylthiocholine is hydrolysed in the embryo, the subembryonic liquid and the yolk. No activity could be detected in the albumen. In the subembryonic liquid the enzymatic activity remained almost constant from day 3 to day 6. Before day 3 not enough liquid has been formed to measure the activity.

The enzymatic activity in the yolk is comparatively high. Furthermore, the enzyme is already active in the yolk of unfertilized and fertilized eggs before

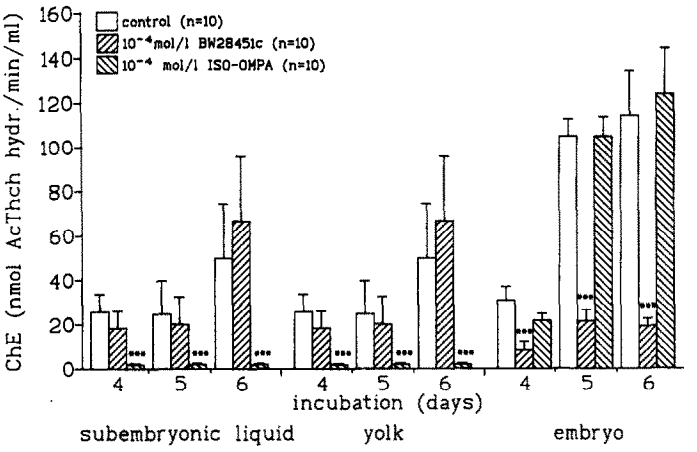


Fig. 2. Cholinesterase (ChE) activity in the subembryonic liquid, yolk and embryo under the influence of BW284c51 and ISO-OMPA. The inhibitors were added to the reaction mixture. Their inhibitory capacity was determined with commercially available preparations of AChE and BuChE as described in Materials and Methods. AcThch hydr. = acetylthiocholinejodide hydrolysed.

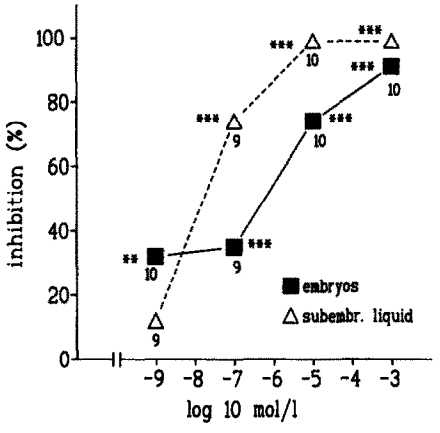


Fig. 3. The influence of various concentrations of dichlorphos on cholinesterase activity in the subembryonic liquid and embryo in proportion to that in control preparations. Dichlorphos was dissolved in 0.9% NaCl and 100 µL of the solution were injected into the subembryonic liquid on day 3 of incubation. One hour later the cholinesterase activity was assessed. Controls were treated with 100 µL of 0.9% NaCl. **P < 0.01, ***P < 0.001 compared with untreated eggs.

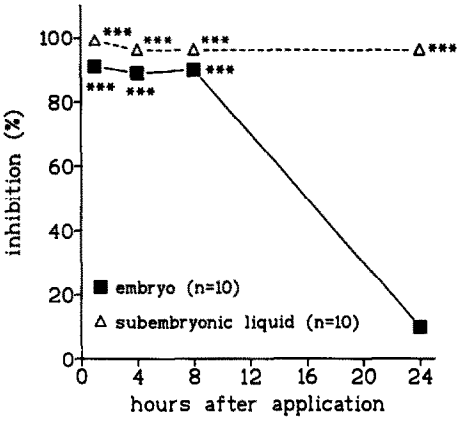


Fig. 4. Time course of cholinesterase inhibition by dichlorphos ($10^{-3}$ M). The conditions were as described in Fig. 3.

incubation is started. A significantly lower activity is attained during the first week of incubation. In contrast to the subembryonic liquid and yolk, the embryonic activity increases in the course of the first week.

In further experiments the enzyme was characterized in more detail. BW284c51 depressed significantly the embryonic enzyme activity but not the extraembryonic enzyme activity. The reverse is true for ISO-OMPA (Fig. 2). Based on these findings the extraembryonic enzyme is a BuChE type, whereas the embryonic enzyme represents AChE.

A different behaviour between the AChE activity from the embryo and the BuChE activity from the subembryonic liquid was also observed when the egg was treated with dichlorphos. The agent administered into the subembryonic liquid blocked both the activity in the subembryonic liquid and in the embryo during the course of 8 hr (Figs 3 and 4). However, the amount of inhibition in the embryo decreased from 91% 1 hr after substance administration to 10% 24 hr after treatment, whereas in the subembryonic liquid, the enzyme remained practically inactivated (Fig. 4).

As a rule, organisms die from organophosphorus intoxications within a short period of time. This does not seem to happen in our experiments. As Fig. 5 shows, only 30–40% of the embryos die within the first 24 hr, when treated with $10^{-3}$ or $10^{-5}$ mol/L.

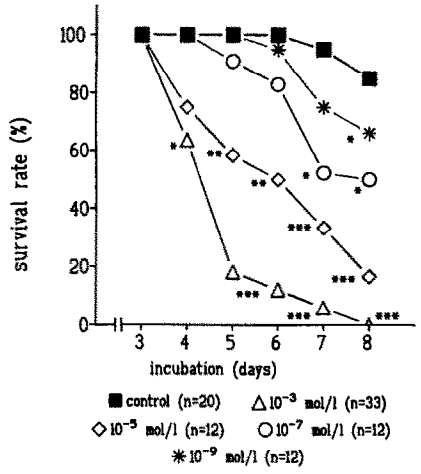


Fig. 5. The effect of dichlorphos on survival rate. Conditions were similar as those described in the legend to Fig. 3. *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control group.

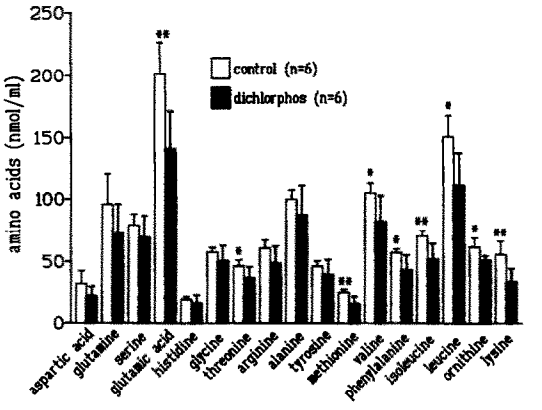


Fig. 7. The influence of dichlorphos on the amino acid content of the subembryonic liquid; 100 μL of $10^{-3}$ M dichlorphos were injected into the subembryonic liquid and 24 hr later the concentration of the amino acids was recorded.

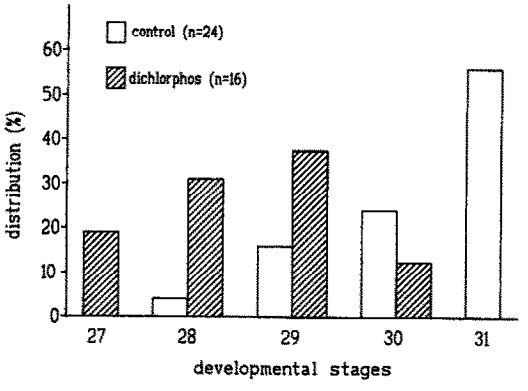


Fig. 6. The influence of dichlorphos on embryonic development; 100 μL of a $10^{-3}$ M dichlorphos solution were injected into the subembryonic liquid on day 3 of incubation and the embryonic stages were recorded on day 6 of incubation.

Most of the embryos died at later stages, when the enzyme activity in the embryos has already been restored (Fig. 4).

Therefore, the question arises of whether there are other toxic effects caused by dichlorphos. In a final series of experiments we followed developmental and metabolic parameters under the influence of dichlorphos. The agent exerted a very pronounced effect on the body weight on day 4 which is 24 hr after treatment. The wet weight was significantly diminished from 58.1 ± 6.4 mg in the control group to 45.2 ± 9.6 mg in the experimental group. Furthermore, the developmental stages were reduced under these conditions. Untreated embryos reached a developmental status between 27 and 29, whereas that of the treated embryos was between stage 29 and 31 (Fig. 6).

As to the metabolic parameters, the glucose content in the subembryonic liquid decreased from 4.6 ± 1.1 μmol in the control group to 3.4 ± 1.1 μmol in the experimental group. Also the concentration of amino acids in the subembryonic liquid was significantly diminished after administration of dichlorphos (Fig. 7).

## DISCUSSION

It is obvious from the studies reported here that much cholinesterase activity is deposited by the hen into the yolk. This is in accordance with the data of Saeed *et al.* [14], who were able to show that the yolk of unfertilized eggs contain two different types of cholinesterase. During the first 3 days of incubation a significant decrease in activity was observed. This decrease in activity may, at least in part, be due to transfer of activity from the yolk into the subembryonic liquid, which is formed from day 3 of incubation onwards [15].

In fact, compared with the embryo, the enzyme in these two compartments is characterized by a similar behaviour. It means there is, in contrast to the activity in the embryo, no significant change in the extraembryonic enzymatic activity between day 3 and day 6 of incubation. Furthermore, ISO-OMPA depressed the cholinesterase activity in the yolk and the subembryonic liquid, whereas no activity changes were observed under the influence of BW284c51. The reverse is true for the enzyme in the yolk and the subembryonic liquid as well. On the basis of these different inhibitory effects released by BW284c51 and ISO-OMPA, respectively, the enzyme in the yolk and in the subembryonic liquid may represent the BuChE-type of enzyme and that in the embryo the AChE-type [16]. This does not, however, mean that in the embryo no cholinesterase other than AChE is found since low activities of cholinesterases which are more or less identical to the BuChE are detectable in muscle [17] and in

blood and liver [14] of chick embryos. Obviously, AChE represents just the predominant cholinesterase type in the embryo.

The increase of AChE in the embryo from day 3 to day 6 is obviously due to its continuous synthesis by the embryo. This is in accordance with the fact that AChE activity, when inhibited by an organophosphorus ester like dichlorphos, has been regenerated within 24 hr after the treatment. This was not the case for BuChE in the subembryonic liquid. Obviously, *de novo* synthesis of the enzyme does not occur in the subembryonic liquid. This would be reasonable if the enzyme originates exclusively from the laying hen.

Surprisingly, AChE activity was not intimately correlated with the survival rate of the embryos. In our experiments, the embryonic enzyme activity has been blocked at least for 8 hr; most of the embryos, however, died at a later stage, when activity has been restored again. In humans a reduction of the activity below 20% of the normal level is critical and leads to death within a few minutes [18]. As the suppression of the cholinergic function of AChE represents the real cause when organisms die from organophosphorus insecticides, one may suggest that this function in cholinergic transmission is of subordinate importance at this developmental stage.

This raises the question as to the function of cholinesterase in the embryo and in the extraembryonic compartments. According to previous reports, AChE and BuChE support the embryonic development in many respects [1–3]. They appear to be important for the regulation of cleavage divisions and are implicated in the regulation of morphogenetic cell movements in the early embryonic stage. This development status, however, was not included in the experiments the paper is dealing with.

Further, cholinesterases seem to be involved in the regulation of transport processes [19]. In this context, it is interesting to note that in our experiments a reduction of glucose and of the amino acids in the subembryonic liquid has been observed under the influence of dichlorphos. Since this happened extraembryonally, one may suggest that an inhibition of BuChE leads to a reduced transport capacity. First of all this may be true for glucose which is known to be transported from the albumen to the subembryonic liquid [15].

In the case of the amino acids, it is still not completely understood how they are made available in the subembryonic liquid. It is very likely that they are derived from yolk proteins. However, no specific proteases have been detected in the yolk so far, but on the other hand cholinesterases are known to degrade protein [20, 21]. If this is true for the yolk cholinesterase then one would expect that an inhibited BuChE would lead to the decrease in the amino acid level. However, further experiments will be needed to elucidate such a direct effect. In any case, retarding the supply of glucose and amino acids as shown in our experiments may not be lethal to the embryo, but may just lead to retardation in development as has been observed.

There have been several reports [4–6, 16] indicating that blocking the cholinesterase leads to malformations. Such an effect has not been seen in our experiments. It should, however, be mentioned that no systematic histological analyses were performed. Furthermore, malformations caused by organophosphorus insecticides become evident at later developmental stages [4–6] of the avian embryo.

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