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BRAIN ACHE OF THE TELEOST CLARIAS LAZERA AS INDICATOR OF WATER CONTAMINATION BY PHENOL OR METHANOL

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ABSTRACT

The present investigation revealed the effect of phenol or methanol at different sublethal concentrations on the specific activity of brain acetylcholinesterase. The study was also extended to include the total protein concentration during the tested period. Phenol has marked excitable effects on the fish even at fairly low concentration (0.05 mg/l) as recorded by activation of AChE in brain. During exposure to methanol the enzyme activity responds in triphasic manner, first activation followed by inhibition then recovery to control level. It seems that the initial activation represents the alarm reaction to the presence of methanol in the environment and during which more nerve impulse transfer may be needed. The effect of methanol was dose-dependent being high with the higher concentration. Methanol may be more harmful than phenol. The potentiality of using the change in the enzyme activity as criterion for water quality was discussed.

INTRODUCTION

ACHE is a ubiquitous enzyme found in various fish tissues including brain, liver, gonads, blood, gut, spleen and muscle (Augustinsson, 1959; Hogan and Knowles, 1968; Matton and Laham, 1969; Hogan, 1971 and Silver, 1974). The relative significance of AChE in nervous tissue or other nerve-integrated tissues fish is apparent in view of the important role of the enzyme in neural transmission (Rosenberry, 1975). The enzym is, however, curiously found in the blood of many fish although no functional role has been attributed to it in this tissue (Hogan, 1971; Gluth and Hanke, 1983 and Hanke et al., 1982). The dilemma is further confused by the fact that it is found in various blood fractions of different fish species (Silver, 1974). Rainbow trout, carp (Cyprinus carpio), tench (Tinca tinca) and eel (Anguilla anguilla), for example are void of AChE activity in the erythrocytes although some esterase activity was present in the serum (Close et al., 1957). In addition, Hogan (1971) observed that AChE was present in the plasma of catfish (Ictalurus spp), grass pickerel (Esox americanus vermiculatus) and northern pike (Esox lucius).

Although the measurement of acetylcholinesterase activity in fishes has proved valuable in detecting pollution of fresh and estuarine water by organophosphate and carbamate insecticides (Williams and Sova, 1966; Holland et al., 1967; Carter, 1971; Coppage and Duke, 1971; Morgan et al., 1973; Livingston and Goodwin, 1974 and Coppage and Braidech, 1976) little is known about the potentiality of using the changes in AChE activity as indicator of stress caused by industrial effluent contaminated by phenol or methanol.

MATERIAL AND METHODS

EXPERIMENTAL ANIMAL:

The fish Clarias lazera were used as experimental animals (Length, 15-20 cm; weight. 40-60 gm). They were purchased from the market in batches of 200. Before they were used for experiments, fish were kept in glass 400-liter aquaria at a stocking density of 10 kg fish per aquarium for at least two weeks. The aquaria received a continuous supply of tap water (temp. 25 $\pm$ 1°C). A photoperiod of 16 L : 8 D was maintained. Dissolved oxygen was never below 90% saturation. Acidity was monitored regularly (pH 7.3 $\pm$ 0.4). Fish were fed at libitum every other day, feeding was interrupted 24 hr before the experiments. Two days prior to the application of chemicals, fish were transferred into the well aerated 20 liter test glass aquaria in proportion of 8 fish per aquarium. During that time and during the later experiments the fish were not fed and the water was carefully renewed every 12 hr. Handled controls were subjected to the same amount of disturbances as the experimental fish, including transfer to another aguaria but they remained in pollutant-free medium.

EXPERIMENTS:

These were initiated by the addition of chemicals to the test aquaria to have final concentration of 1.0, 0.1 and 0.05 mg phenol/liter or 100 and 1 ml/liter methanol. The fish were killed in groups of 8, except otherwise stated, at various times up to 7 days after transfer to test aquaria (3, 6, 9, 24, 48 and 168 hr). Sacrifices were made at the same time of the day to preclude the potential effects of diurnal fluctuation on enzyme activities.

BRAIN SAMPLE:

Fish were caught by hand net and quickly anaesthesized in MS 222 solution (66.7 mg/l, tricaine methane sulfonate, Syndel Labs.) in a separate aquarim. Immobilization was complete within 20 seconds. Brain was dissected and directly frozen.

Acetylcholinesterase (AChE) Assay:

Brain was weighted and quickly sonicated in 2 ml of 100 mM phosphate buffer at pH 4.2, using an ultrasonic power

with cooling during and between each period of sonication. The temperature of the homogenate was not allowed to rise above 4°C. The homogenate was centrifuged in cold centrifuge at 22000 c for 20 min. The supernatant was transferred to clean test tube immersed in ice bath and analysed for enzyme activity. Acetylcholinesterase activity was measured via the reaction of the colorimetric reagent 5,5' -dithio-bis-2-nitrobenzoic acid (DTNB) with the product of acetylthiocholine (ASCh) hydrolysis (Ellman et al., 1961; Voss and Sachsse, 1970 and Hussain, 1979). ASCh (173.5 mg/ml) was prepared daily in pH 8 phosphate buffer. DTNB (39.6 mg) was dissolved in 9.75 ml pH 7.0 phosphate buffer to which 250 ml of sodium bicarbonate (60 mg/ml) were added (Ellman et al., 1961). The DTNB solution is stable for 2 to 3 days if stored at 4°C.

All assay of AChE activity were performed in duplicate or triplicate at 24°C and employed 2.8 ml homogenate solution to which 100 ul DTNB and 50 ul ASCH solutions were added. ASCh concentration in the cuvettes was 10 mM and corresponded to the substrate level for maximum activity (Ellman et al., 1961 and Hobden and Klaverkamp, 1977). An additional preparation without ASCh served as the blank. The change in absorbance initiated by the addition of ASCh was monitored from 1 to 5 min. on Baush and Lomb spectrophotometer (Model Spectronic 2000) equipped with a chart recorded at a wavelength of 512 nm. Nonenzymatic hydrolysis was determined by substituting 2.8 ml of pH 8.0 phosphate buffer for homogenate solutions. Reproducibility of the assay technique was verified from determinations of AChE activity from a standard AChE enzyme extract (Sigma Chemical Co.)).

Total AChE activities were expressed as nmol ASCh hydrolysed per minute calculated from the change in absorbance per min., given an extinction coefficient of 1.36 x 10^4 $M^{-1}cm^{-1}$. Brain tissue enzyme specific activity is given in terms of unit/mg protein (Lowry et al., 1951).

STATISTICAL ANALYSIS:

Data were analysed using students t-test. Statistical significance is judged on overlap of 95% confidence intervals ($p<0.05$). Values in tables are expressed as mean $\pm$ standard error of the mean (S.E.M.), while in figures, percentage of changes versus controls (100%), are primarily demonstrated. If there was a significant deviation of the experimental from the control means, the columns in the figures were marked with an arrow.

RESULTS

Brain Acetylchloinesterase (AChE):

The alterations in AChE specific activity after treatments with phenol are presented in Fig. 1. A clear significant ($p<0.02$) inhibition of the enzyme activity was

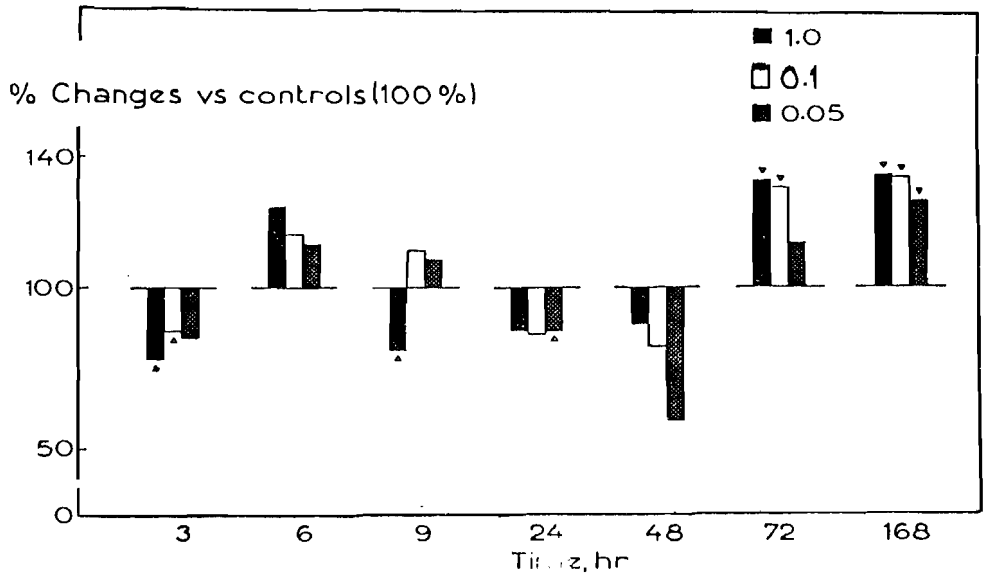

FIG .1.
Changes of brain AChE specific activity level in percentage of controls during 168 hr of exposure to 1.0, 0.1 and 0.05 mg/L phenol.

measured in fish exposed to the two higher phenol concentrations during the first 3 hr. The severity of inhibition was dose-dependent being higher with the higher concentration (ca. 22%). After this initial phase of inhibition, a recovery to normal control level was observed more or less by exposure from hr 6 till the 48th hr at all concentrations. Thereafter and the enzyme was significantly activated by exposure up to hr 72 throughout the rest of the experimental time. The level of the increase in enzyme specific activity was also dose-dependent.

During the first 9 hr of exposure to both concentrations of methanol, the AChE specific activity was higher in the brain of treated fish than that of control ($p<0.001$). At the 10 ml/liter methanol the activation of the enzyme increased with time reaching a maximum after 9 hr. A recovery to normal control level at both concentrations were recorded toward the end of experiment after an intermediate phase by exposure hr 24 and 48 during which the enzyme was significantly inhibited ($p<0.02$). The magnitude of the inhibition and that of the recovery was not dose-dependent (Fig. 2).

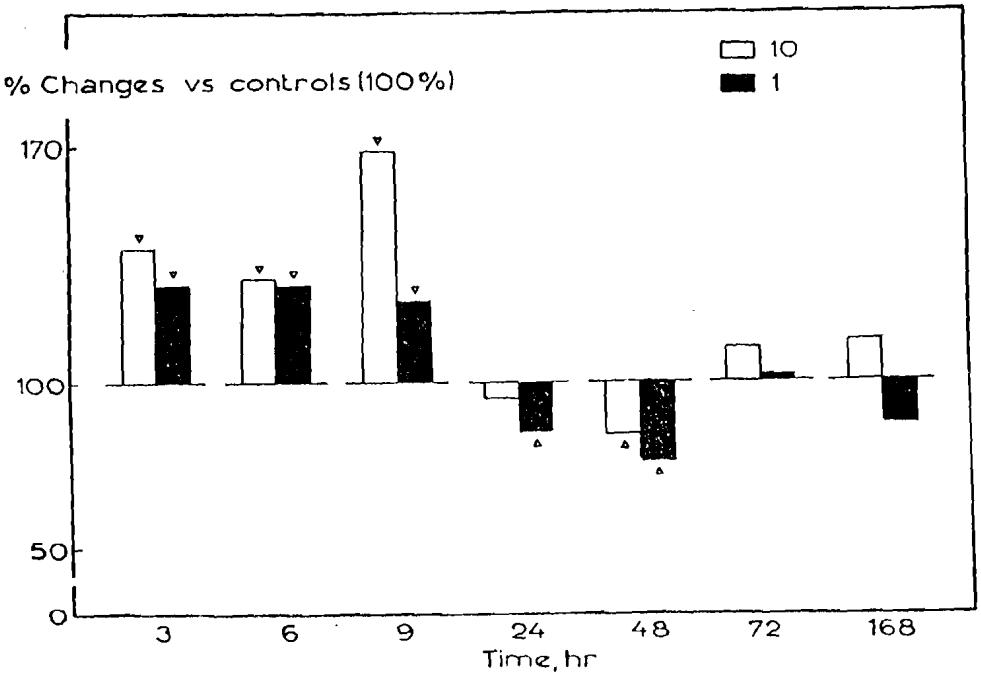

FIG .2.
Changes of brain AChE activity level in percentage of controls during 168 hr of exposure to 10 and 1 ml/L methanol.

Protein Concentration:

During exposure to either phenol (Table 1) or methanol (Table 2), the changes of protein contents were variable and non of the increase or decrease was statistically significant when compared to the respective controls.

DISCUSSION

A number of studies have indicated the importance of the physiological and biochemical changes in the central nervous system of poikilotherms during different causes of stress, and the possible limiting role of the central nervous system (CNS) in the overall process (Roots and Prosser, 1962; Prosser and Farhi, 1965; Hussein et al., 1983; Hanke et al., 1982; Gluth and Hanke, 1983 and Assem, 1984 & 1985).

In fish, as mammals, brain acetylcholinesterase (AChE, EC 3.1.1.7) was investigated by many authors (e.g. Hanke et al., 1982 and Assem, 1984 and 1985) as the enzyme responsible for terminating the action of the neurotransmitter, acetylcholine (ACh), on the acetylcholine receptor (AChR) (Eccles, 1964 and Katz, 1966).

TABLE .1.
Changes of brotein in the brain during exposure to different phenol concentrations.

Experimental conditions	1.0 mg/L Mean	S.E.M	0.1 mg/L Mean	S.E.M	0.05 mg/L Mean	S.E.M.
Control (3hr)	2.4	±0.23				
3 hr	3.1	±0.16	3.0	±0.15	2.8	±0.19
Control (6hr)	3.3	±0.18				
6 hr	3.2	±0.20	3.4	±0.15	3.7	±0.17
Control (9hr)	3.1	±0.17				
9 hr	3.6	±0.28	2.9	±0.18	2.9	±0.17
control (24hr)	3.2	±0.22				
24 hr	2.8	±0.24	2.8	±0.19	2.6	±0.18
Control (48hr)	2.9	±0.24				
48 hr	2.4	±0.14	2.3	±0.24	3.6	±0.29
Control (72hr)	2.6	±0.23				
72 hr	2.5	±0.08	2.4	±0.06	2.7	±0.09
Control (168hr)	3.0	±0.16				
168 hr	2.9	±0.23	2.7	±0.17	3.1	±0.20

Protein : mg/100 mg.

TABLE .2.
Changes of brotein in the brain during exposure to two methanol concentrations.

Experimental conditions	10.0 ml/L Mean	S.E.M.	1.0 ml/L Mean	S.E.M.
Control (3hr)	3.20	±0.04		
3 hr	3.40	±0.28	3.36	±0.06
Control (6hr)	3.20	±0.04		
6 hr	3.30	±0.079	3.40	±0.13
Control (9hr)	3.20	±0.04		
9 hr	3.40	±0.17	3.28	±0.05
Control (24hr)	2.73	±0.03		
24 hr	2.76	±0.22	2.74	±0.14
Control (48hr)	2.73	±0.03		
48 hr	2.90	±0.12	2.81	±0.19
Control (72hr)	2.80	±0.03		
72 hr	2.64	±0.11	2.62	±0.11
Control (168hr)	2.62	±0.03		
168 hr	2.76	±0.13	2.76	±0.06

Protein : mg\100 mg.

In our studies, and during exposure to phenol, brain AChE specific activity was shortly inhibited by exposure 3 hr only at the two higher phenol concentrations (1.0 and 0.1 mg/liter). After a rapid recovery to corresponding controls level, which extended to exposure 48 hr a highly significant activation of the enzyme units was recorded throughout the rest of experimental time at all concentrations. These results indicate that phenol has marked excitable effects on the fish at fairly low concentration. These excitable effects of phenol are thought to be due to a generalized facilitation of synaptic transmission (Banna and Jabbur, 1970). In our experiments, we changed the water of the aquarium every 12 hr (15% decrease of phenol concentration) together with the low solubility of phenol in water (80 g/liter) and its low degree of dissociation at physiological pH (Kaila and Saarikoski, 1980), we would expect that the excitable effects are caused by the nonionized forms of the compounds, this is in agreement with the results obtained by Kaila and Saarikoski (1980). Assem (1985) has also found an excitable effects of phenol represented by increased brain AChE activity, when he subjected the carp Cyprinus carpio to the same phenol regime. But our results differ from his (Assem, 1985) in two aspects, firstly in carp the effects of phenol were mainly restricted to the higher concentration (1.0 mg/l) and secondly recovery to normal control level was observed by him after 168 hr. These differences suggested that our experimental fish Clarias lazera could be more sensitive to phenol than the carp, which might restrict its choice as a candidate in aquaculture.

During exposure to methanol, the enzyme activity (u/mg pr) responds in a triphasic manner, a first activation (3, 6 and 9 hr) followed by an inhibition of activity (24 and 48 hr) then recovery to control level during the rest of exposure time (73 to 168 hr). It seems that the initial increase of the enzyme activity represents the alarm reaction to the presence of methanol and during which more nerve impulse transfer may be needed. The effects of the higher methanol concentration (10 ml/liter) were more pronounced than the effect of the lower concentration (1.0 ml/liter) especially by 9 hr. A similar initial increase of brain AChE activity was found in the sheepshead minnow, Cyprinodon variegatus, exposed to low concentrations of the organophosphate insecticide Diazinon (Goodman et al., 1979) and in the carp, Cyprinus carpio, exposed to DDT (Assem, 1984).

The inhibition of the ACHE activity recorded by exposure 24 and 48 hr at both methanol concentrations may represent the resistance phase during which the fish tries to regain homeostasis. Resistance led to compensation as represented by the subsequent (from 72 hr) recovery of AChE activity to normal control level. These changes were not dose-dependent which contradict the assumption made by Weiss (1961) who stated that the length of time required for recovery of AChE activity to normal, following exposure to intoxication, is dependent on the extent of previous inhibition and its duration.

Comparing the results of exposure to phenol and methanol, we would concluded that the resistance led to compensation at the given concentrations of methanol but to further resistance at all phenol concentrations, as seen by the activation of AChE activity from exposure 72 hr onwards. These different AChE reactions between methanol and phenol support the assumption that methanol may be more harmful than phenol at their investigated concentrations.

To our knowledge nothing have been published concerning the effects of methanol on brain AChE of fish which make the mechanism of its mode of action difficult to explain. Its activation effects may be like phenol due to an increase in transmitter liberation from presynaptic terminals (Otsuka and Nonomura, 1963 and Kuba, 1969), while its inhibition effects may be like the effects of organophosphate poisoning in mammals which putatively involve antagonizing the effect of ACh on AChR (Hayes, 1975).

The absence of any significant changes in brain protein concentration throughout the whole exposure time to either phenol or methanol indicates that all measured changes in AChE activity were true and not due to changes in brain water content.

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