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Comparison of Selected Parameters for Monitoring Methoxychlor-induced Hepatotoxicity[1]

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The purpose of the present investigation was to demonstrate the relative sensitivities of some commonly used toxicity tests and some lesser known biochemical assays in demonstrating sublethal liver damage in the rat following oral administration of low levels of an organochlorine insecticide. It was decided to employ a common insecticide with low intrinsic toxicity and some degree of organ specificity. Methoxychlor (MC) meets these basic requirements. MC is practically non-toxic to animals (oral LD_{50} in rats, 6000 mg/kg) and is slowly metabolized by microsomal enzymes in mammals by pathways similar to those for its congener DDT. MC would be expected to cause some degree of hepatotoxicity, by virtue of being an organochlorine compound, as well as its structural similarity to DDT which can cause characteristic liver damage (HAYES 1959).

The parameters commonly used to monitor hepatotoxicity, which were compared in this investigation, were obtained through two enzyme assays and two histochemical techniques. Measurement of circulating levels of serum glutamic-pyruvic transaminase (SGPT) is considered to be one of the most sensitive indicators of liver damage and serum glutamic-oxalacetic transaminase (SGOT) is nearly as sensitive (STREET 1970). GRICE et al. (1971) showed that with diethylaminoethanol-induced hepatotoxicity, SGOT levels were elevated prior to histologic evidence of necrosis (detected by light microscopy), but with CCl_4-induced hepatotoxicity, early histologic signs of hepatic involvement could be seen at doses not affecting SGOT levels. It therefore seemed logical to incorporate SGOT and SGPT in a study involving detection of MC-induced hepatotoxicity. Since the most common changes observed in the liver following administration of organochlorines are fatty accumulation and necrosis, liver sections were stained with hematoxylin and eosin (H&E) as well as sudan black B (fat stain).

The remainder of the parameters compared in this work relate to enzyme activities involved in liver glycogen metabolism. The assays include those for glycogen levels, glycogen synthetase activity, glycogen phosphorylase activity, and glucose-6-phosphatase activity. Lactate and pyruvate levels, though widely used as

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measures of "oxygen debt" (HUCKABEE 1958) and mitochondrial redox state (KREBS et al. 1969), also relate to gluconeogenesis and therefore liver function (SMITH et al. 1953) and are included in the present investigation.

Preliminary studies in our laboratory indicate that induction or inhibition of enzyme activity related to glycogen metabolism in rat liver is at or near maximum level 24 h after the acute administration of either DDT or MC. Serum transaminase levels should also be maximal in 24 h, judging from work reported by CORNISH (1971), in which SGOT and SGPT were maximally elevated in 24 h after CCl_4 administration to rats.

MATERIALS AND METHODS

Thirty five male Holtzman rats, weighing 200 to 250 g, were housed in 5 group cages, acclimated to the laboratory environment as well as to oral intubation, minimizing stress. Rats were randomly assigned to five groups of seven to attain the same mean weight per group. On the day prior to sacrifice, all animals received 16 mL/kg body wt of pure corn oil, or 10, 40, 160, or 640 mg/kg methoxychlor (90% pure Grade II; Sigma Chemical Co.) in corn oil, administered orally. One additional rat was treated with undiluted carbon tetrachloride (p.o., 2.5 mg/kg) to serve as a positive control for histologic evaluation (GIGNOLI & CASTRO 1971). All handling and dosing was begun at noon and the animals were sacrificed 24 h after dosing.

In order to further minimize stress, which could immediately affect glycogen levels as well as some enzyme activities, the animals were anesthetized with sodium pentobarbital (75 mg/kg, i.p.) prior to sacrifice. Upon exposure of the abdominal cavity, approximately 1 g of the median lobe of each liver was removed and quickly frozen between two slabs of dry ice. These tissue samples were stored at -70°C with all subsequent cutting and weighing performed in a -20°C cold room. About 2 mL fresh whole blood was obtained by heart puncture. The blood samples were placed on ice, allowed to clot, and centrifuged; the serum obtained was stored at 0-5°C for less than one week. Liver samples for histologic evaluation were taken from the tissue immediately adjacent to the origin of the first sample.

SGOT and SGPT levels were determined according to the Sigma Technical Bulletin 55-UV except for the following changes. Rather than using a 3 mg% solution of potassium dichromate for zeroing the spectrophotometer as suggested for human serum, solutions of 6 mg% and 11 mg% were used for SGPT and SGOT blanks, respectively.

Liver glycogen levels were measured by the method of HASSID & ABRAHAM (1957). Glycogen phosphorylase activity was assayed in the direction of glycogen synthesis using a modification of the method employed by GILBOE et al (1972). The homogenizing solution for the phosphorylase assay was that described by GILBOE & NUTTAL (1972), and the glycogen was purified by the method of

THOMAS et al. (1968). Total glycogen synthetase and synthetase I activities were assayed by the method described by THOMAS et al. (1968). Glucose-6-phosphatase activity was assayed by the method of SWANSON (1955). Liver pyruvic and lactic acid levels were measured by the methods outlined in the Sigma Technical Bulletin No. 726-UV and 826-UV for blood, after modification to allow these determinations in tissue. About 250 mg tissue was homogenized in 1.0 mL $HClO_4$ (0.8%) and decanted into a centrifuge tube. The homogenizer tube was then rinsed with 0.5 mL of the homogenizing solution, and the combined solutions were centrifuged (12500 x g, 10 min, 2°C). The clear, protein-free supernate was removed and stored at 0-5°C until assayed. For the pyruvate determination, 3.3 rather than 2.2 mL of 1.5 M Trizma base solution was used to prepare the NADH reagent, since failure to neutralize the excess acid results in denaturation of the lactic dehydrogenase. For the lactate determination, the above-mentioned, protein-free supernate was substituted directly for serum.

Statistical significance was determined by analysis of variance using the Duncan multiple range test (NIE et al. 1975).

RESULTS

The activities of synthetase I and total synthetase were calculated, as well as the synthetase I/total ratios for each group of animals. No significant changes were noted for these parameters when compared to control values. Statistical analysis also showed that no significant rise in the levels of either transaminase resulted from MC administration at the doses used. Liver glycogen levels exhibited a dose-related decrease with each increase in dose of MC and a total decrement to about 75% of control value at the highest dose. However, since the decrease was gradual, the Duncan multiple range test failed to show significant differences between groups. (The decrement at the highest dose was significantly different from control values at the .05 level, using the Student t test.)

A significant increase in the level of the microsomal enzyme, glucose-6-phosphatase, occurred at the highest dose of MC (Figure 1). Also shown in Figure 1 are the values for glycogen phosphorylase which decreased significantly at the two highest doses. Liver pyruvate levels did not change significantly at any dose used, and there was no significant change in the pyruvate/lactate ratios. Lactate levels, on the other hand, dropped significantly at all but the lowest dose of MC (Figure 1).

Histological examination of liver sections after staining with H&E showed no differences between control livers and those of MC-treated animals. It should be noted that fatty accumulation is difficult to discern, using this histologic stain even in the positive control. For this reason, the fat stain was used. The liver sections from treated rats had the same nominal amount of fat as the corn oil controls, and only the positive CCl_4 controls exhibited fatty accumulation.

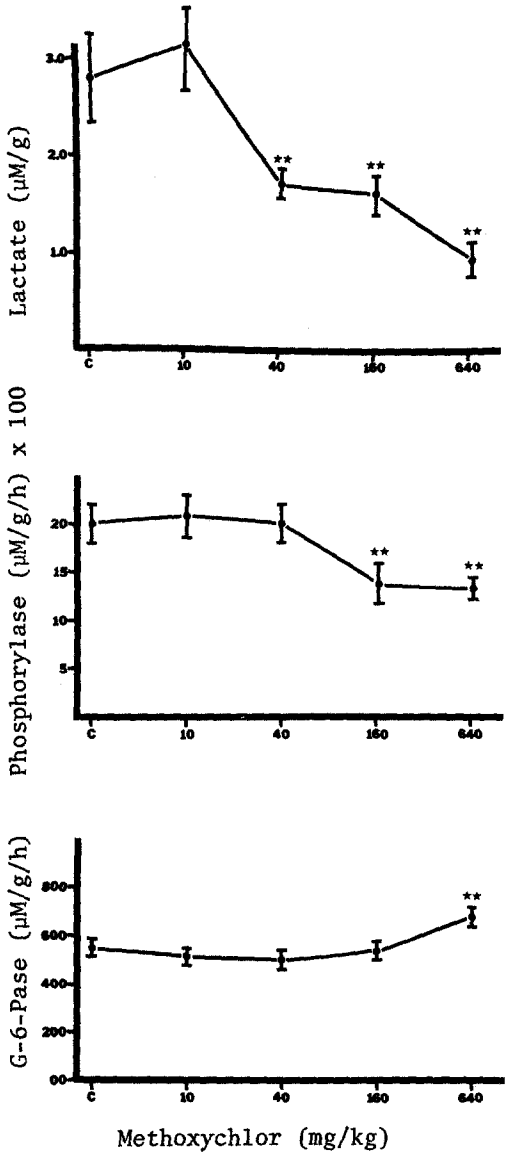

Figure 1. Hepatic lactate, glycogen phosphorylase, and glucose-6-phosphatase levels of control and MC-treated rats, 24 h after oral dosing. Mean ± SE; N=7. **Differs significantly from control, $p \leq 0.01$.

DISCUSSION

In this study we have attempted to focus on the biochemical changes occurring in liver glycogen metabolism 24 h after oral administration of very low doses (relative to the LD_{50}) of a chlorinated insecticide which is fairly specific for that organ. The exact mechanism of toxicity of MC remains unclear and is beyond the scope of this paper. This investigation was designed to determine which assays were sensitive to low levels of toxicity, and as such, generated a fair amount of negative data.

In this case, standard assays such as serum transaminases, pyruvate/lactate ratios, and histological staining were not sensitive enough to detect organ damage. The more sensitive assays consisted of those for monitoring liver lactate, glucose-6-phosphatase, and glycogen phosphorylase, with that for lactate being the most sensitive. Liver lactate levels decreased significantly after a dose of MC which was less than 1% of the oral LD_{50} (rat).

From a theoretical standpoint, it is not surprising to find that enzymes associated with glycogen metabolism can be utilized as markers of subtle hepatotoxicity, since they mediate one of the primary functions of the liver. Similar sensitivities of these same enzymes have been seen in this laboratory with DDT after i.p. injection in rats. Perhaps these parameters can be used to monitor sub-lethal changes in the liver caused by low doses of other pesticides and hepatotoxic substances.

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