HLTH-1

STRATEGIES FOR STUDYING THE IMPACT OF MULTICHEMICAL EXPOSURE TO HAZARDOUS CHEMICALS: IMPLICATIONS FOR HEALTH RISK ASSESSMENT

Final Report for Year 1: September 30, 1985

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PERSONNEL

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OVERVIEW

The overall goal of this research is to develop strategies for evaluating the toxicity of complex mixtures of chemicals, such as those encountered in toxic waste exposures.

Objectives for Year 1

1. To conduct a workshop with invited experts who have gained experience working with mixtures in order to discuss the relative merits and disadvantages of possible strategies
2. To establish criteria which should be satisfied in the design of the experimental investigations
3. To select and analyze the mixtures for use in initial studies and to identify the toxic actions and target organs for these mixtures
4. To determine the ability of the selected mixtures to alter the toxicity of known toxic substances
5. To assess the predictability of the data obtained from the work above

Progress to Date

1. The workshop was held on December 10, 1984. A summary of the presentations and discussions at this meeting was previously submitted.
2. Criteria were established for the design of experimental studies. These criteria were summarized in the revised grant submitted in January, 1985.
3. Three chemicals, in various mixtures, were selected for initial studies. These were carbon tetrachloride, monochlorobenzene, and lead acetate.
4. Toxicity studies with the mixtures were carried out on rats, data collected, and analyzed. The results of these studies are summarized in the Abstract (below) and in more detail in the Progress Report.
5. Assessment of the predictability of this data is an ongoing process.

ABSTRACT

Mixtures grant

First Year Report - September, 1985

The animal studies conducted during the first year of this grant consisted of subchronic studies of toxicity of single chemicals, synthetic mixtures of these chemicals and a leachate sample. As planned, we are using two approaches to this complex problem: (1) evaluation of the toxicity of synthetic mixtures, i.e. looking for possible synergistic effects as opposed to additive or antagonistic effects and (2) evaluation of the effects of a modified environmental toxic sample (from a toxic waste site) on the toxicity of a known compound.

Our major thrust was on the synthetic mixtures. The initial mixture contained carbon tetrachloride (CCl_4), monochorobenzene (MCB) and lead acetate (PbAc). These compounds were selected because they are found in most toxic wastes throughout this state and the entire United States and are renal and hepatic toxins. Lead compounds are also demonstrated hematotoxins. The doses picked were of necessity sufficiently low so that no one compound would completely inhibit any enzyme system (or produce a maximal measurable toxic effect on a system), thus allowing detection of possible synergistic effects. The toxicities of the single compounds have been widely studied but there are no data on exposure to combinations of these chemicals. The compounds were administered orally to mature Sprague-Dawley rats for a period of one week at three dose levels (X, 2X and 4X concentrations), both as the single compounds and as multiple combinations of two and three chemicals (for detailed protocol, see Progress Report). All experiments were carried out six times with one animal/group in each experiment so that any random effects were minimized. Statistical evaluation included the use of the Student t test and two way anaylsis of variance using a SAS program.

Toxic effects were indeed observed in the predicted range in the expected target organs. Tests for specific tissue damage as well as tests for general toxicity showed that one rarely gets additive effects of these compounds, at least at the concentrations administered. For example, lead acetate at all doses increased the kidney/body weight ratio while CCl_4 partially antagonized this gain, despite the fact that CCl_4 alone had no significant effect (at the low dose level).

In contrast, alkaline phosphatase levels were increased following CCl_4 treatment but not with either PbAc or MCB treatment while the mixture of all three had less of an effect than that of CCl_4 alone.

Studies of the mutagenicity of the environmental sample using *Salmonella typhimurium* strains TA 98 and 100 gave negative results despite the fact that analysis indicated that the sample contained mutagenic chemicals.

Abstract
Mixtures Grant
Future Work

In future work (year 2) we plan to continue our studies with synthetic mixtures. We will use more complex mixtures, prepared by addition of other chemicals, one at a time, to existing mixtures. These chemicals include: chromic oxide, nickel (Ni^{+2}) chloride, PCBs (Aroclor 1254), toluene and trichloroethylene. The toxicity tests currently in use will be used to evaluate possible interactions occurring with these more complex mixtures (see Experimental Design, p 23, for specific details.

In years 2 and 3, we will also study the effect(s) of environmental mixtures (such as modified leachates) on the toxicity of the known toxins, lead acetate and carbon tetrachloride (previously studied) and the carcinogen, dimethylnitrosamine (DMN) (see below).

We will also initiate new and unique research using a highly sensitive technique, that of post-labeling analysis of carcinogen-DNA adducts, using ^{32}P. The formation of specific DNA-adducts has been correlated with the carcinogenic potential of some chemicals. This post-labeling technique permits the labeling of these adducts after their formation, either *in vivo* or *in vitro*. This labeling allows the rapid, sensitive detection and identification of these adducts (at levels previously undetectable). DNA (containing the adducts) is partially purified and then digested to nucleotides and a ^{32}P labelled PO_4 group is substituted for the cold 5' PO_4. After being labelled, the adducts are separated and identified by thin layer chromatography or HPLC and subsequent autoradiography. This new technique has been applied to several carcinogens, including DMN, so that well-characterized adducts are available as standards. DMN is of interest to us as a known carcinogen.

This technique will be used in two ways in our research: (1) to study effects on the pattern of adduct formation by the known carcinogen, DMN, following oral administration of both the DMN and a synthetic mixture (2) detection and identification of components of a mixture which form adducts following administration of the mixture. Comparison with published standards or standards prepared by incubation of the single components of the mixture with purified DNA will make this identification possible. (See Experimental Design, p 25). This technique has great potential for identification and characterization of DNA-adducts formed after exposure of experimental animals to complex environmental mixtures. The method eliminates the need for costly labelling of the many individual components of a synthetic mixture to detect reaction products with DNA.

Mutagenicity studies will be carried out on urine samples collected from rats administered the modified leachate samples or the synthetic mixtures. This technique detects the mutagenic *effects* of any metabolites excreted in the urine. The urine will be concentrated on an XAD column and aliquots tested for mutagenic activity with *Salmonella typhimurium* strains which detect base apri and frame shift mutagens. Testing of soluble urine samples lessens the probability of binding of samples to the bacterial cell walls or to the support agar in the assay, a problem encountered in studying the leachate directly.

Progress Report
Mixtures Grant

I Introduction

The specific aims for the first year of this grant were accomplished to a large extent. We held a workshop, as planned, and consulted experts who have worked with mixtures; a report of that meeting was included in our first Progress Report. We also (1) selected several synthetic mixtures for investigation, and (2) identified the toxic actions and target organs of toxicity of the selected mixtures. We are currently addressing the problem of aim (3) which was to determine the potential of selected mixtures to alter the toxicity of known substances. Aim (4) to assess the predictability of the data obtained is an ongoing process.

During the first year of this grant our emphasis was on toxicity studies with synthetic and natural (leachate) mixtures to detect synergistic or antagonistic actions. We used synthetic mixtures to allow us to better predict resulting toxicity as well as the interactions which might occur. Chemicals included in the synthetic mixture were selected as representative of compounds which have been found in leachate and drinking water samples. The leachate was from a local waste site. The design and results of these studies are discussed below.

II. Experimental Design.

The first synthetic mixture studied contained lead acetate (PbAc), carbon tetrachloride (CCl_4), and monochlorobenzene (MCB). These chemicals were administered orally, both separately and in various combinations, at three dose levels to mature male Sprague-Dawley rats (120-140g) daily for one week. (The detailed protocol for these studies is outlined in Table 1). Lead acetate was administered in water solution while CCl_4 and MCB were each given in corn oil solution. In these studies, several indices of toxicity were measured. These indices are listed in Table 2.

Mutagenicity studies were also carried out with a modified environmental sample (from a waste site) using <u>Salmonella typhimurium</u> strains TA 98 and TA 100 obtained from Dr. Bruce Ames. These bacterial strains were specifically developed to mutate to prototrophy in the presence of base pair and frame shift mutagens. The samples were dissolved in DMSO and were tested for the presence of direct acting mutagens (without metabolic activation) at several dose levels using the plate incorporation method of Ames <u>et al.</u> (Mut, Res, <u>31</u>, 365-380, 1975). The sample was tested at 3 dilutions (1:1,1:5 and 1:10). Toxicity studies were also carried out to determine at what level the sample was toxic to the bacteria, as tests are not carried out on concentrations of any samples which cause > 80% cell death.

All data from the synthetic mixture study were subjected to two way analysis of variance with the use of a SAS program. The Student t test is used for analysis of the mutation studies, although in general a value of twice background is considered positive in the bacterial assay. These results were all equal to background, so that they were not subjected to any such analysis.

III. Results.

A. Synthetic Mixtures

1. Body Weight Changes

All animals which were treated with the highest dose of the single chemicals or with the mixture of the highest doses of the three chemicals showed decreases in body weight gain compared to control animals (Figure 1). When animals received CCl_4 either alone or in combination with MCB or PbAc (all at lower doses), there were also decreases in weight gain vs control gains. The mixtures of CCl_4/MCB and CCl_4 /PbAc caused greater decreases than treatment with only CCl_4; the effect of MCB with CCl_4 appeared to be synergistic (greater than additive).

2. Organ/Body Weight Ratios

In general an alteration in organ/body weight (organ/bw) ratios following exposure to chemicals indicates that adaptive or adverse effects have occurred in the organ. Tables 3-4 show the organ/body weight ratios for liver, kidney, testes, lung, adrenal glands and spleen. Most organ/body weight ratios were not affected by treatment either with the chemicals alone or with the various mixtures, with some important exceptions. As shown in Figure 2, the liver/bw ratios were increased with single doses of both CCl_4 and MCB, as well as following administration of a mixture of the two, which produced additive effects. Treatment with the triple mixture caused increases in ratios which were significantly different from those following treatment with MCB and from control values but which did not differ from ratios from CCl_4-treated animals. The effects were most pronounced at high doses.

Administration of PbAc caused a significant increase in kidney/bw ratio (Figure 3); this was partially antagonized by CCl_4 and MCB. Although treatment with all of the chemicals alone and in the 4 mixtures caused slight increases in the testes/bw ratios (Figure 4), only the mixture of CCl_4 and MCB caused increases that were statistically significant. In this case, these compounds appeared to act synergistically. There were no sigificant changes in the kidney-, spleen- or adrenal organ/body weight ratios with any of the above treatments.

3. Hematological Studies

Results (available to date) of hematological studies have not indicated treatment-related changes. Hematocrit values and white cell counts were similar in all groups. These data are summarized in Table 5. Differential white cell counts are currently being measured.

4. Clinical Chemistry Studies

Results of clinical chemistry analyses are shown in Tables 6 and 7. Following administration of the individual chemicals, only CCl_4 caused a slight elevation of liver enzymes in serum (Table 6). Increases in alkaline phosphatase (Figure 5) and in glutamic-pyruvic transaminase (SGPT) activities (Figure 6) were found after treatment with CCl_4 alone (see also Table 6). All CCl_4-containing treatments caused some elevation in both alkaline phosphatase and SGPT activities; the effect on alkaline phosphatase was

greater than that on SGPT, however. as the effect on GPT was evident only with log transformation of the data. MCB had no effect on CCl_4-induced elevations in alkaline phosphatase activities, or any effect on either enzyme activity when given alone.

None of the compounds or mixtures caused a significant change in blood urea nitrogen (BUN) values (Table 7;Figure 7), although PbAc had been shown to cause increased kidney/bw ratios, a different indication of kidney damage.

Blood glucose concentrations were also all within normal limits. Slight (but not significant) lowering of blood glucose followed admdinistration of the highest dose of the mixture of the three compounds. (Table 7).

As seen in Figure 8, delta-aminolevulinic acid dehydratase (ALAD) activity in red blood cells was significantly lowered in all groups receiving any PbAc (Table 7). Administration of the mixture of CCl_4 and MCB also significantly lowered the activity of this enzyme but did not add to the decrease caused by PbAc when this mixture was given along with the PbAc. Treatment with CCl_4 also had a slight but significant depressing action on this enzyme. MCB treatment alone had no effect on this enzyme at any level.

Cytochrome P-450 levels were depressed to approximately 50% of controls in the livers of animals given MCB alone. Although the assays are not completed on all 144 samples from the mixtures study,it is clear that the MCB depressed this heme enzyme more than did ingestion of PbAc or CCl_4, both of which have ben shown to depress this enzyme at high concentrations. When the data are collected there may be an emergence of a clear pattern for this enzyme system. Concentrations of the other heme protein, cytochrome b_5, were not affected by any of the treatments in these tests. Results from the determinations of cytochrome C reductae are not yet calculted.

5. Histological data are not yet available.

B. Leachate

There was no mutagenic effect with this sample obtained from a waste site as the number of revertants for bacteria exposed to the samples was not greater than for bacteria exposed to the vehicle. The sample was mildly toxic at 1:1 dilution as 50% of the bacteria survived, and was non-toxic at greater dilutions. The low toxicity of the sample combined with the lack of mutagenicity suggests that the compounds are not getting through the bacterial cell wall or the bacterial membrane. Protein binding studies are in progress to determine whether the compounds are binding to the support agar on the plates.

IV. Discussion.

The chemicals selected as components of the first synthetic mixtures study are not only known components of environmental toxic mixtures, but also the mechanisms of toxicity of these compounds are well defined. For example, it is known that lead salts react with -SH groups in proteins (and other molecules) and Pb^{+2} compounds are known to depress the activity of δALAD, an -SH-containing enzyme found both in liver and in red blood cells, and one of

the enzyme activities monitored in these studies. It is possible that an epoxide metabolite from MCB could also inhibit -SH enzymes by a similar mechanism. Carbon tetrachloride is metabalized in hepatic microsomes to a free radical (CCl_3.) which should act locally and not affect the ALAD activity in red cells. The observed effect of CCl_4 on this enzyme could be secondary to other biochemical actions. An advantage of the use of known chemicals in the mixture studies is that indications of the mechanisms of action and interaction can be derived from the final results. That is, if two compounds interfere with the activity of an enzyme by the same mechanism, this pathway may become saturated and a maximum effect can be reached. In contrast, if two compounds act by different mechanisms the final results may be seen to be synergistic.

The negative results of the mutagenicity studies reinforce past studies in these laboratories on mixtures (incuding a leachate from a N.J. landfill site) in which samples of the mixtures gave negative results in the same S. thyphimurium bacterial systems used in these assays. Whether the compounds bind to the bacterial cell wall and thus prevent entry of any mutagenic moieties, or whether there is non specific-binding of the compounds to the support agar on which the bacteria are grown is not known. Collection of urine from animals given these mixtures is a procedure which should allow detection of mutagenic metabolites formed from any of the constituents. (This can be carried out during the second year of the grant; see Future studies).

The dose of CCl_4 was sufficient to cause increases in SGPT and alakaline phosphatase activities, both indications of hepatic damage, but there was no apparent decrease in cytochrome P-450 in microsomes from CCl_4 treated animals (data not shown). Other workers have found that daily administration of relatively low doses of CCl_4 causes regeneration of the MFO enzymes. This may be the phenomenon which we are seeing with these rats. CCl_4 was chosen for our work as a representative alkyl halogenated compound about which there are many data points. This type of response may be found with many compounds.

Table 1

Experimental Design for Study of the toxicity of lead acetate, monochlorobenzene, and carbon tetrachloride

Group 1 vehicle	Group 9 vehicle	Group 17 vehicle
Group 2 low dose A	Group 10 medium dose A	Group 18 high dose A
Group 3 low dose B	Group 11 medium dose B	Group 19 high dose B
Group 4 low dose C	Group 12 medium dose C	Group 20 high dose C
Group 5 low dose A + low dose B	Group 13 medium dose A + medium dose B	Group 21 high dose A + high dose B
Group 6 low dose A + low dose C	Group 14 medium dose A + medium dose C	Group 22 high dose A + high dose C
Group 7 low dose B + low dose C	Group 15 medium dose B + medium dose C	Group 23 high dose B + high dose C
Group 8 low doses of A + B + C	Group 16 medium doses of A + B + C	Group 24 high doses of A + B + C

All groups consist of 6 rats each. All compounds and mixtures of compounds are administered per os daily for seven days. The animals are killed and autopsied on the eighth day.

Compound A, lead acetate, is dosed at 13.7, 27.5, and 55 mg/kg.

Compound B, carbon tetrachloride, is dosed at 0.125, 0.25, and 0.5 ml/kg.

Compound C, monochlorobenzene, is dosed at 75, 150, and 300 mg/kg.

Table 2

Toxicity Tests used in Evaluation of Synthetic Mixtures

1. Body weight measurement at initiation of study, daily during the study and at exposure termination

2. Hematologic tests (hematocrit, red and white cell counts, differential counts) to assess effects on the hematopoietic system at termination of the exposure

3. Clinical blood chemistries (in plasma or blood: SGPT, alkaline phosphatase, urea nitrogen, glucose) to evaluate liver and kidney function at termination of the exposure

4. Neurobehavioral screening at termination of the exposure

5. Necropsy, organ weight measurements, gross signs of organ damage, and histopathological evaluation of selected tissues at termination of the exposure

6. Preparation of liver microsomes and assay for cytochrome P-450, cytochrome b_5, and NADPH cytochrome c reductase

TABLE 3

Organ/ Body Weight Ratios

Treatment	Dose mmol/ kg	Liver/ Body Weight	Kidney/ Body Weight	Testes/ Body Weight
Vehicle	--	0.0449 ± 0.0038	0.00895 ± 0.00058	0.0106 ± 0.0021
PbAc	0.036	0.0470 ± 0.0047	0.01045 ± 0.00088	0.0106 ± 0.0004
CCl_4	1.30	0.0526 ± 0.0035	0.00873 ± 0.00043	0.0107 ± 0.0013
MCB	0.666	0.0518 ± 0.0054	0.00954 ± 0.00065	0.0107 ± 0.0016
PbAc + CCl_4	0.036 1.30	0.0517 ± 0.0048	0.00957 ± 0.00086	0.0107 ± 0.0006
PbAc + MCB	0.036 0.666	0.0492 ± 0.0027	0.00963 ± 0.00053	0.0105 ± 0.0010
CCl_4 + MCB	1.30 0.666	0.0507 ± 0.0042	0.00892 ± 0.00091	0.0109 ± 0.0008
PbAc + CCl_4 + MCB	0.036 1.30 0.666	0.0532 ± 0.0043	0.00947 ± 0.00069	0.0108 ± 0.0009
Vehicle	--	0.0449 ± 0.0038	0.00895 ± 0.00058	0.0106 ± 0.0021
PbAc	0.072	0.0456 ± 0.0020	0.01048 ± 0.00055	0.0107 ± 0.0008
CCl_4	2.60	0.0508 ± 0.0055	0.00840 ± 0.00052	0.0108 ± 0.0012
MCB	1.332	0.0509 ± 0.0044	0.00884 ± 0.00056	0.0106 ± 0.0017
PbAc + CCl_4	0.072 2.60	0.0529 ± 0.0031	0.00936 ± 0.00079	0.0117 ± 0.0015
PbAc + MCB	0.072 1.332	0.0507 ± 0.0020	0.00943 ± 0.00097	0.0108 ± 0.0017
CCl_4 + MCB	2.60 1.332	0.0557 ± 0.0041	0.00929 ± 0.00025	0.0118 ± 0.0014
PbAc + CCl_4 + MCB	0.072 2.60 1.332	0.0585 ± 0.0046	0.00976 ± 0.00065	0.0111 ± 0.0011
Vehicle	--	0.0449 ± 0.0038	0.00895 ± 0.00058	0.0106 ± 0.0021
PbAc	0.144	0.0454 ± 0.0048	0.01073 ± 0.00063	0.0116 ± 0.0009
CCl_4	5.20	0.0575 ± 0.0061	0.00967 ± 0.00136	0.0117 ± 0.0016
MCB	2.664	0.0547 ± 0.0044	0.00906 ± 0.00055	0.0114 ± 0.0009
PbAc + CCl_4	0.144 5.20	0.0525 ± 0.0050	0.00967 ± 0.00066	0.0123 ± 0.0014
PbAc + MCB	0.144 2.664	0.0544 ± 0.0030	0.01032 ± 0.00033	0.0112 ± 0.0013
CCl_4 + MCB	5.20 2.664	0.0580 ± 0.0047	0.00984 ± 0.00073	0.0136 ± 0.0016
PbAc + CCl_4 MCB	0.144 5.20 2.664	0.0588 ± 0.0090	0.00999 ± 0.00085	0.0124 ± 0.0009

TABLE 4

Organ/ Body Weight Ratios (cont'd)

Treatment	Dose mmol/ kg	Adrenals/ Body Weight	Spleen/ Body Weight	Lung/ Body Weight
Vehicle	--	0.000185 ± 0.000026	0.00475 ± 0.00104	0.00574 ± 0.00073
PbAc	0.036	0.000184 ± 0.000061	0.00461 ± 0.00082	0.00565 ± 0.00031
CCl_4	1.30	0.000174 ± 0.000038	0.00385 ± 0.00063	0.00569 ± 0.00069
MCB	0.666	0.000172 ± 0.00090	0.00471 ± 0.00090	0.00636 ± 0.00104
PbAc + CCl_4	0.036 1.30	0.000184 ± 0.000026	0.00417 ± 0.00082	0.00551 ± 0.00065
PbAc + MCB	0.036 0.666	0.000169 ± 0.000042	0.00440 ± 0.00050	0.00559 ± 0.00084
CCl_4 + MCB	1.30 0.666	0.000186 ± 0.000019	0.00466 ± 0.00114	0.00570 ± 0.00156
PbAc + CCl_4 + MCB	0.036 1.30 0.666	0.000197 ± 0.000032	0.00393 ± 0.00041	0.00585 ± 0.00137
Vehicle	--	0.000185 ± 0.000026	0.00475 ± 0.00104	0.00574 ± 0.00073
PbAc	0.072	0.000197 ± 0.000048	0.00454 ± 0.00060	0.00575 ± 0.00042
CCl_4	2.60	0.000174 ± 0.000033	0.00373 ± 0.00090	0.00569 ± 0.00071
MCB	1.332	0.000165 ± 0.000025	0.00423 ± 0.00110	0.00583 ± 0.00039
PbAc + CCl_4	0.072 2.60	0.000180 ± 0.000028	0.00352 ± 0.00059	0.00549 ± 0.00028
PbAc + MCB	0.072 1.332	0.000179 ± 0.000037	0.00451 ± 0.00091	0.00559 ± 0.00028
CCl_4 + MCB	2.60 1.332	0.000220 ± 0.000135	0.00342 ± 0.00091	0.00611 ± 0.00151
PbAc + CCl_4 + MCB	0.072 2.60 1.332	0.000230 ± 0.000052	0.00418 ± 0.00071	0.00658 ± 0.00161
Vehicle	--	0.000185 ± 0.000026	0.00475 ± 0.00104	0.00574 ± 0.00073
PbAc	0.144	0.000214 ± 0.000045	0.00495 ± 0.00064	0.00611 ± 0.00061
CCl_4	5.20	0.000215 ± 0.000042	0.00416 ± 0.00122	0.00636 ± 0.00097
MCB	2.664	0.000164 ± 0.000016	0.00368 ± 0.00076	0.00580 ± 0.00068
PbAc + CCl_4	0.144 5.20	0.000204 ± 0.000023	0.00364 ± 0.00053	0.00598 ± 0.00108
PbAc + MCB	0.144 2.664	0.000173 ± 0.000034	0.00377 ± 0.00062	0.00569 ± 0.00023
CCl_4 + MCB	5.20 2.664	0.000216 ± 0.00030	0.00316 ± 0.00051	0.00609 ± 0.00065
PbAc + CCl_4 + MCB	0.144 5.20 2.664	0.000201 ± 0.000043	0.00370 ± 0.00113	0.00643 ± 0.00143

Table 5

Hematological Parameters

Treatment	Dose mmol/kg	Hematocrit	White Blood Cell Count
Vehicle	--	34.8 ± 3.0	5680 ± 3080
PbAc	0.036	33.5 ± 3.4	5000 ± 2671
CCl_4	1.30	36.1 ± 4.1	6250 ± 1707
MCB	0.666	33.3 ± 4.0	4416 ± 1544
PbAc + CCl_4	0.036 1.30	34.0 ± 7.0	4555 ± 2398
PbAc + MCB	0.036 0.666	31.5 ± 3.2	4681 + 1820
CCl_4 + MCB	1.30 0.666	34.3 ± 2.6	4969 ± 3182
PbAc + CCl_4 + MCB	0.036 1.30 0.666	34.0 ± 3.8	4223 ± 2115
Vehicle	--	34.1 ± 3.8	4583 ± 2664
PbAc	0.072	36.2 ± 4.0	3561 ± 1456
CCl_4	2.60	36.7 ± 3.3	5847 ± 3238
MCB	1.332	35.1 ± 5.9	4208 ± 3905
PbAc + CCl_4	0.072 2.60	36.0 ± 3.6	4667 ± 3166
PbAc + MCB	0.072 1.332	33.7 ± 3.3	4819 ± 2283
CCl_4 + MCB	2.60 1.332	38.0 ± 4.1	5270 ± 2660
PbAc + CCl_4 + MCB	0.072 2.60 1.332	40.3 ± 2.8	4379 ± 2216
Vehicle	--	35.0 ± 3.4	6076 ± 3085
PbAc	0.144	33.2 ± 3.0	6309 ± 3043
CCl_4	5.20	36.8 ± 5.0	5900 ± 2705
MCB	2.664	33.8 ± 2.0	5188 ± 1400
PbAc + CCl_4	0.144 5.20	40.0 ± 4.2	5365 ± 2180
PbAc + MCB	0.144 2.664	34.8 ± 3.5	5125 ± 2127
CCl_4 + MCB	5.20 2.664	36.5 ± 6.8	9546 ± 2995
PbAc + CCl_4 + MCB	0.144 5.20 2.664	36.3 ± 4.0	4820 ± 1251

Table 6

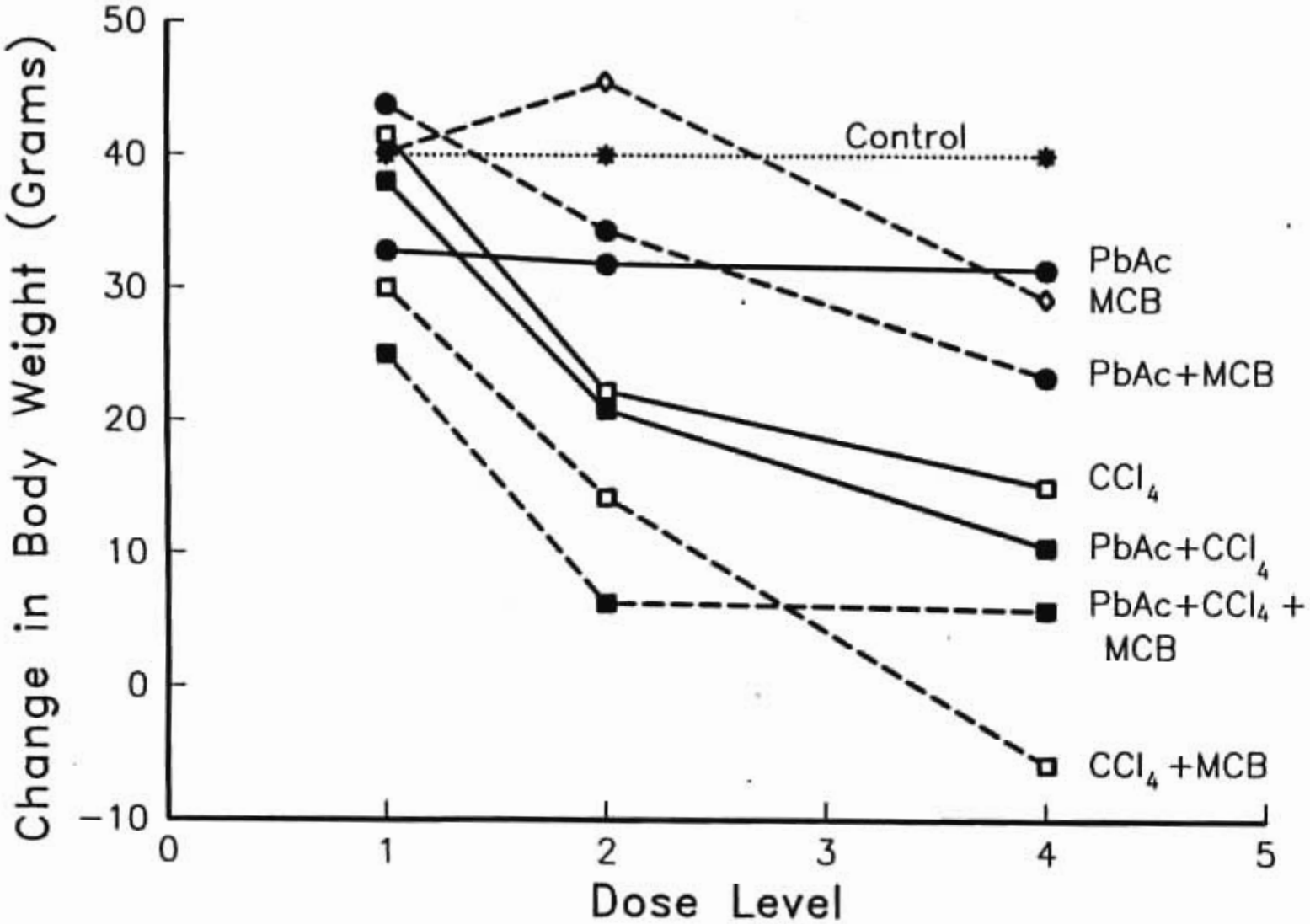
Serum Liver Enzymes

Treatment	Dose mmol/kg	SGPT SFUnits/ml	Alkaline Phosphatase Sigma Units/ml
Vehicle	--	35.4 ± 12.6	9.28 ± 1.94
PbAc	0.036	34.6 ± 6.9	8.97 ± 0.83
CCl_4	1.30	55.8 ± 12.97	9.36 ± 1.37
MCB	0.666	43.5 ± 14.7	7.70 ± 2.11
PbAc + CCl_4	0.036 1.30	47.0 ± 5.4	11.98 ± 2.14
PbAc + MCB	0.036 0.666	44.6 ± 19.4	8.15 ± 1.77
CCl_4 + MCB	1.30 0.666	52.5 ± 8.1	9.28 ± 1.34
PbAc + CCl_4 + MCB	0.036 1.30 0.666	89.3 ± 39.6	10.64 ± 2.18
Vehicle	--	40.8 ± 8.2	8.89 ± 0.53
PbAc	0.072	36.5 ± 15.6	7.31 ± 0.93
CCl_4	2.60	69.5 ± 37.1	12.86 ± 0.52
MCB	1.332	43.0 ± 15.3	8.19 ± 0.74
PbAc + CCl_4	0.072 2.60	97.8 ± 21.5	15.02 ± 1.43
PbAc + MCB	0.072 1.332	45.3 ± 17.7	9.16 ± 2.61
CCl_4 + MCB	2.60 1.332	100.5 ± 36.9	12.83 ± 3.96
PbAc + CCl_4 + MCB	0.072 2.60 1.332	94.7 ± 28.9	14.10 ± 4.35
Vehicle	--	34.0 ± 8.2	6.88 ± 0.96
PbAc	0.144	37.8 ± 13.5	8.80 ± 2.24
CCl_4	5.20	99.5 ± 34.1	16.44 ± 3.96
MCB	2.664	41.8 ± 13.9	7.70 ± 0.74
PbAc + CCl_4	0.144 5.20	96.5 ± 22.6	15.32 ± 4.92
PbAc + MCB	0.144 2.664	44.3 ± 14.8	8.70 ± 0.80
CCl_4 + MCB	5.20 2.664	108.3 ± 20.6	17.36 ± 7.13
PbAc + CCl_4 + MCB	0.144 5.20 2.664	89.5 ± 30.4	12.01 ± 3.69

Clinical Chemistry Analysis

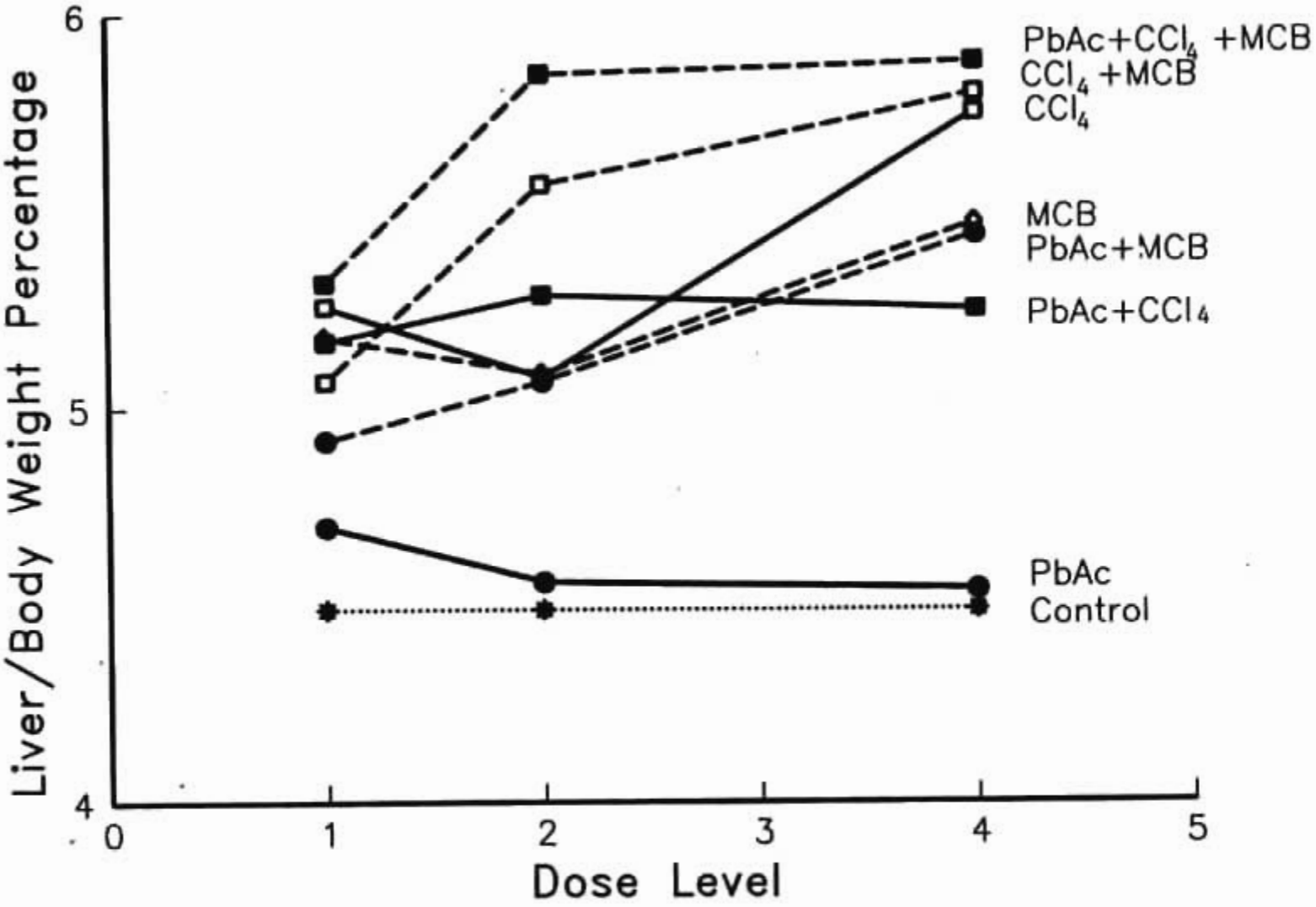
Treatment	Dose mmol/kg	Blood Glucose mg %	Blood Urea Nitrogen mg %	Red Cell ALA-D U/l
Vehicle	--	137 ± 5	36.8 ± 2.7	14.32 ± 5.32
PbAc	0.036	139 ± 7	37.9 ± 5.1	3.72 ± 1.72
CCl_4	1.30	141 ± 17	44.2 ± 5.4	11.54 ± 3.82
MCB	0.666	149 ± 15	38.5 ± 4.8	15.55 ± 2.78
PbAc + CCl_4	0.036 1.30	145 ± 14	39.0 ± 6.4	4.66 ± 1.49
PbAc + MCB	0.036 0.666	154 ± 17	34.8 ± 6.3	4.31 ± 1.18
CCl_4 + MCB	1.30 0.666	132 ± 22	38.3 ± 9.0	10.34 ± 2.87
PbAc + CCl_4 + MCB	0.036 1.30 0.666	131 ± 8	37.0 ± 4.4	4.03 ± 2.50
Vehicle	--	138 ± 7	36.5 ± 3.1	15.64 ± 7.00
PbAc	0.072	138 ± 20	32.9 ± 1.7	3.38 ± 2.62
CCl_4	02.60	142 ± 7	37.8 ± 2.1	8.54 ±3.47
MCB	1.332	143 ± 24	31.6 ± 3.7	17.75 ± 8.67
PbAc + CCl_4	0.072 2.60	137 ± 3	34.8 ± 3.3	3.42 ± 1.98
PbAc + MCB	0.072 1.332	139 ± 9	36.8 ± 5.6	4.79 ± 1.93
CCl_4 + MCB	2.60 1.332	136 ± 26	38.6 ± 3.4	8.72 ± 3.58
PbAc + CCl_4 MCB	0.072 2.60 1.332	124 ± 39	38.2 ± 3.5	2.25 ± 2.00
Vehicle	--	146 ± 19	28.7 ± 2.5	13.19 ± 3.13
PbAc	0.144	149 ± 15	27.9 ± 2.4	2.62 ± 0.70
CCl_4	5.20	146 ± 15	37.6 ± 8.9	8.85 ± 3.36
MCB	2.664	148 ± 14	26.2 ± 6.4	15.02 ± 4.30
PbAc + CCl_4	0.144 5.20	147 ± 12	41.8 ± 8.1	2.43 ± 1.81
PbAc + MCB	0.144 2.664	158 ± 10	32.0 ± 5.6	2.88 ± 1.03
CCl_4 + MCB	5.20 2.664	139 ± 0	37.4 ± 3.4	6.62 ± 7.26
PbAc + CCl_4 + MCB	0.144 5.20 2.664	138 ± 12	44.4 ± 5.3	1.81 ± 1.41

Figure 1



Body weight change = body weight at time of sacrifice - body weight on the first day of feeding.
Administration of all compounds was *per os*, one dose daily, for 7 days.
PbAc (lead acetate) was given in water solution; CCl_4 and MCB (Monochlorobenzene) were given in corn oil.
Doses were as follows: X = low dose; 2X = medium dose; 4X = high dose.
X = 1 on abscissa
X-CCl_4 = 1.30 mmoles/kg body weight
X-PbAc = 0.036 mmoles/kg body weight
X-MCB = 0.666 mmoles/kg body weight

Figure 2

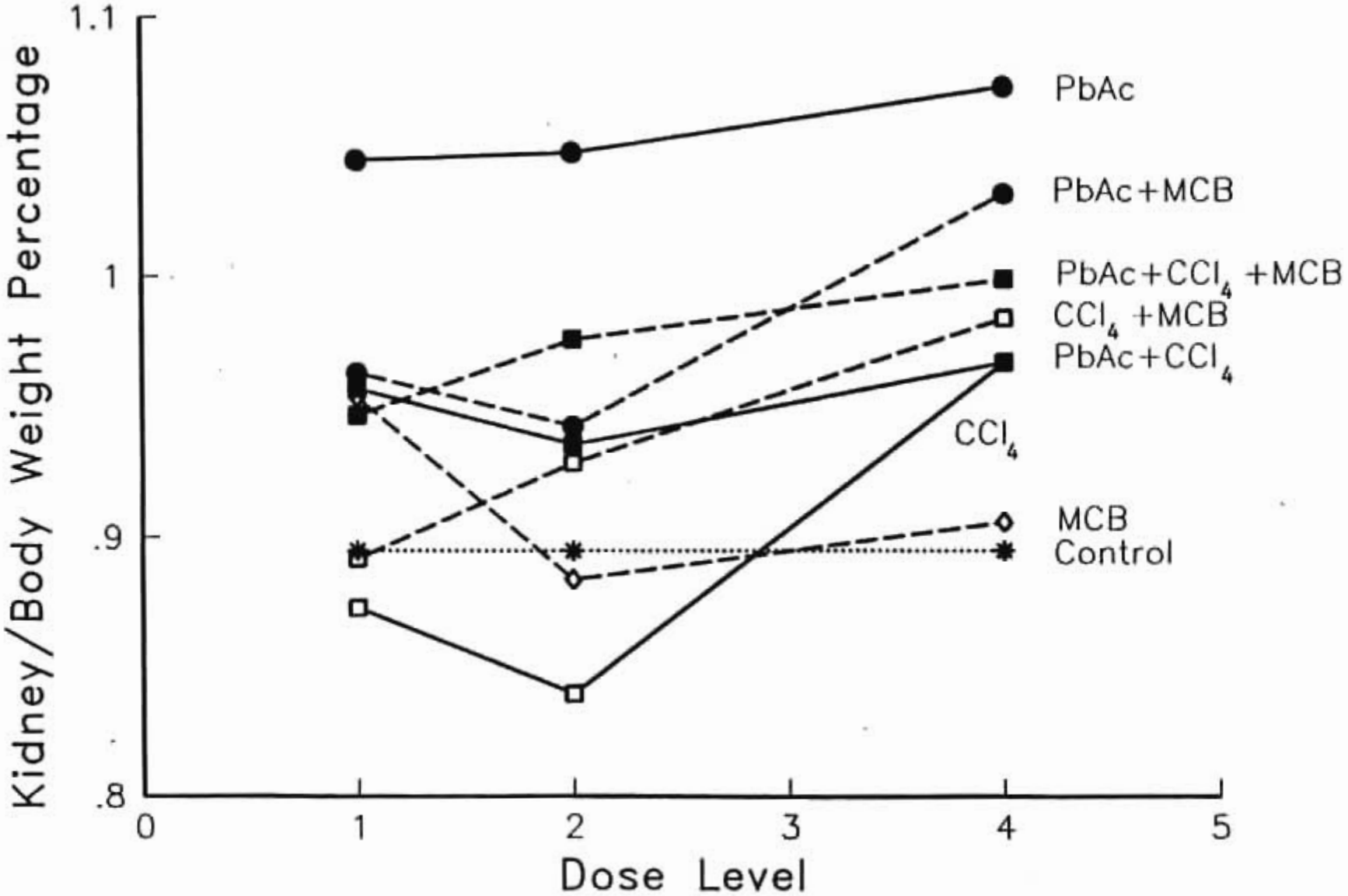


For dose levels and abbreviations, see Figure 1

Administration was *per os*, daily, for one week.

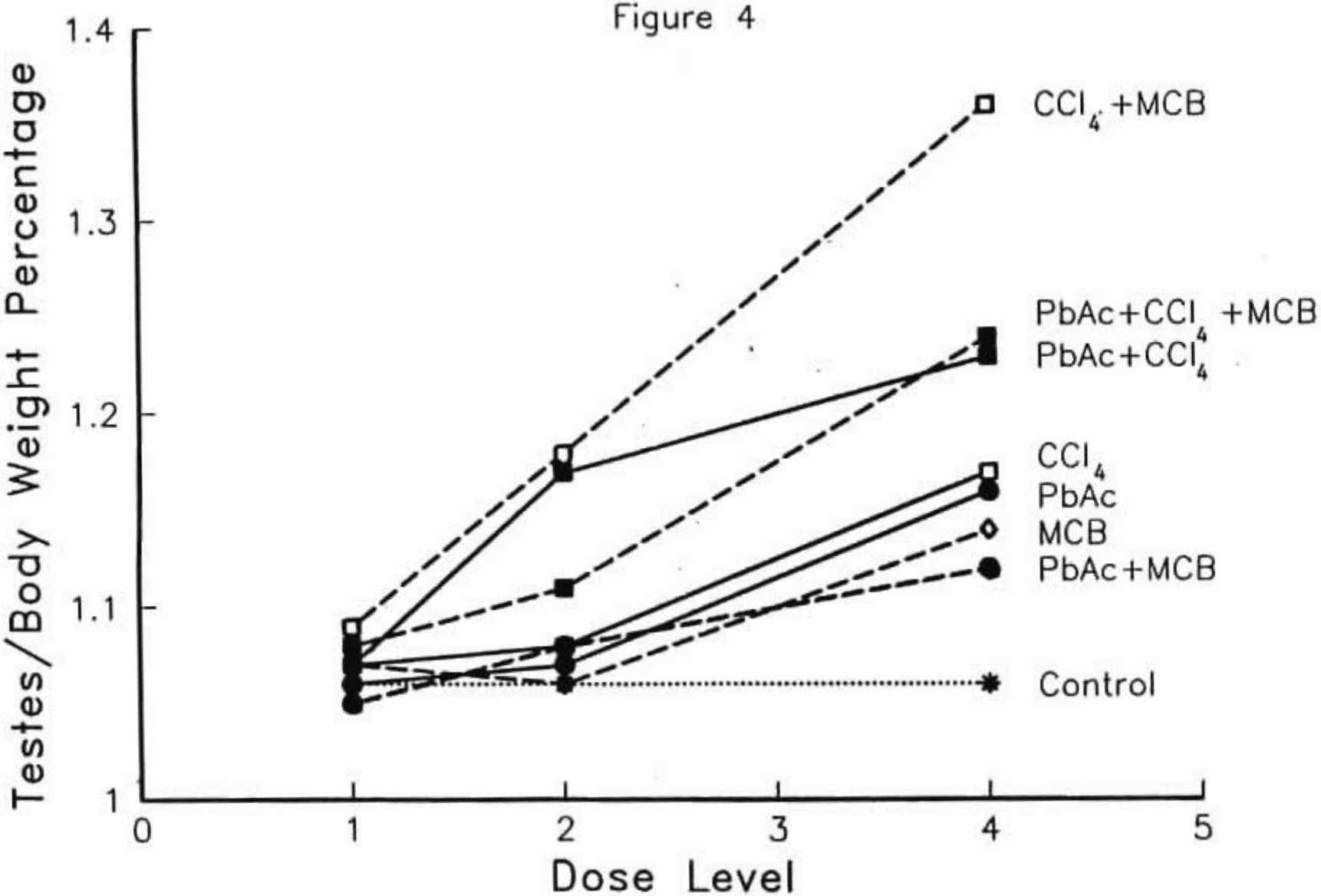
Body Weight is weight at time of sacrifice

Figure 3

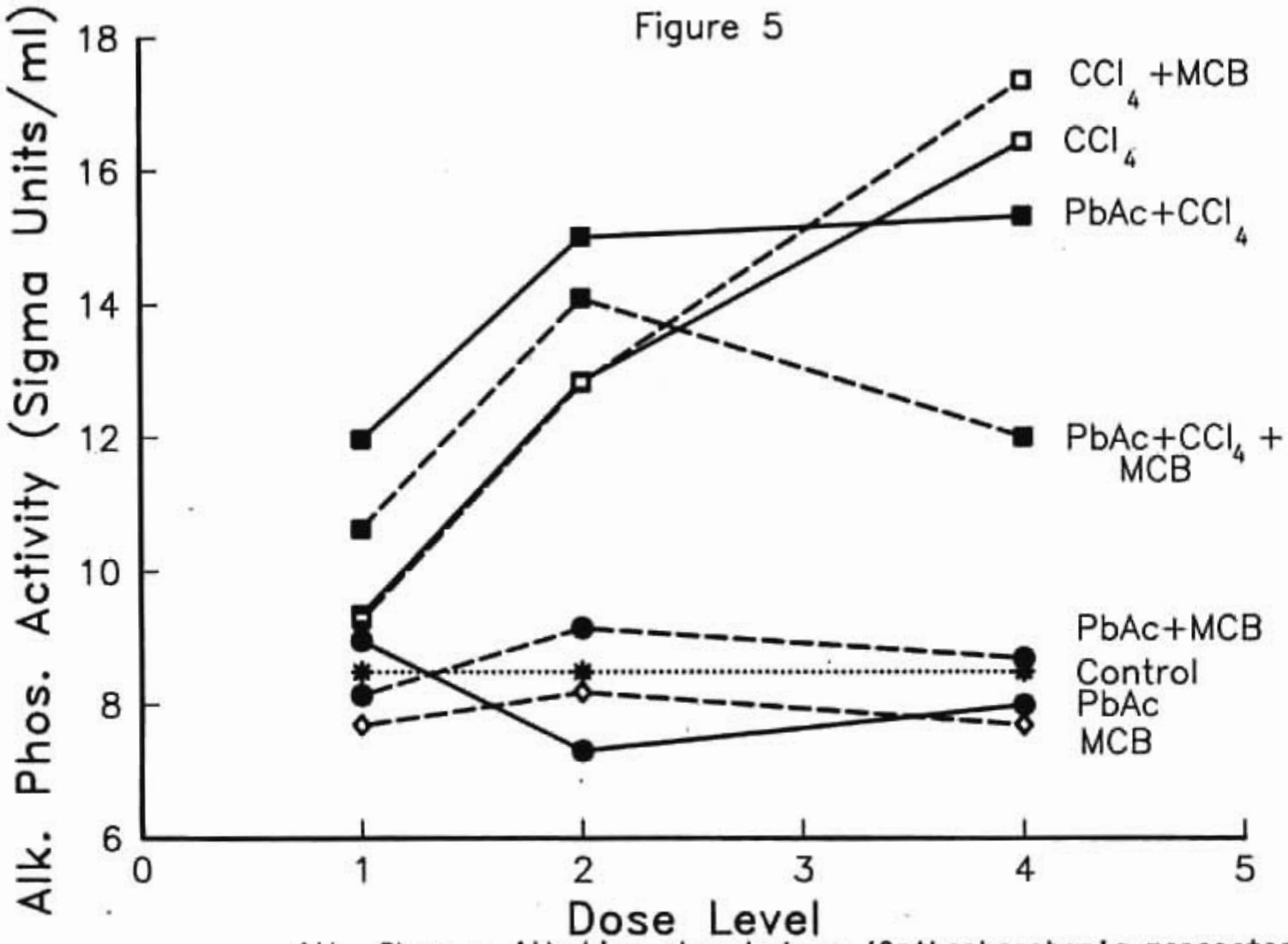


Administration was *per os*, daily, for one week.
Body Weight is weight at time of sacrifice
Administration was *per os*, daily, for 7 days.
For doses and abbreviations, see Figure 1.

Figure 4



Administration was *per os*, daily, for one week.
Body Weight is weight at time of sacrifice
Administration was *per os*, daily, for 7 days.
For doses and abbreviations, see Figure 1.



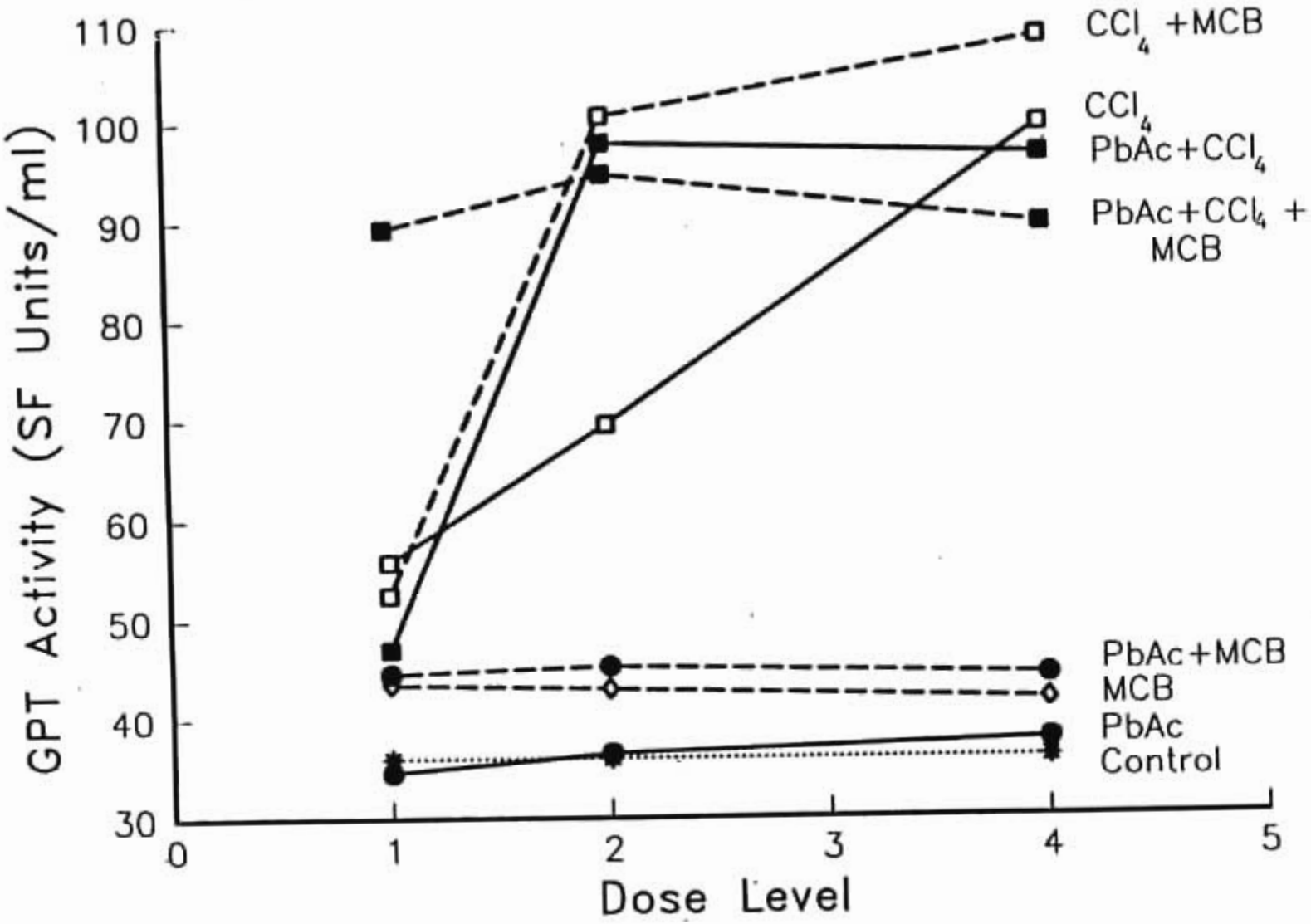
Alk. Phos. - Alkaline phosphatase (Orthophosphoric-monoester phosphohydrolase)

Sigma Unit - one unit will hydrolyze 1.0 umole of p-nitrophenyl phosphate per minute at pH 10.4 at 37°.

Determination of Alk. Phos. activity was carried out using a Sigma Kit

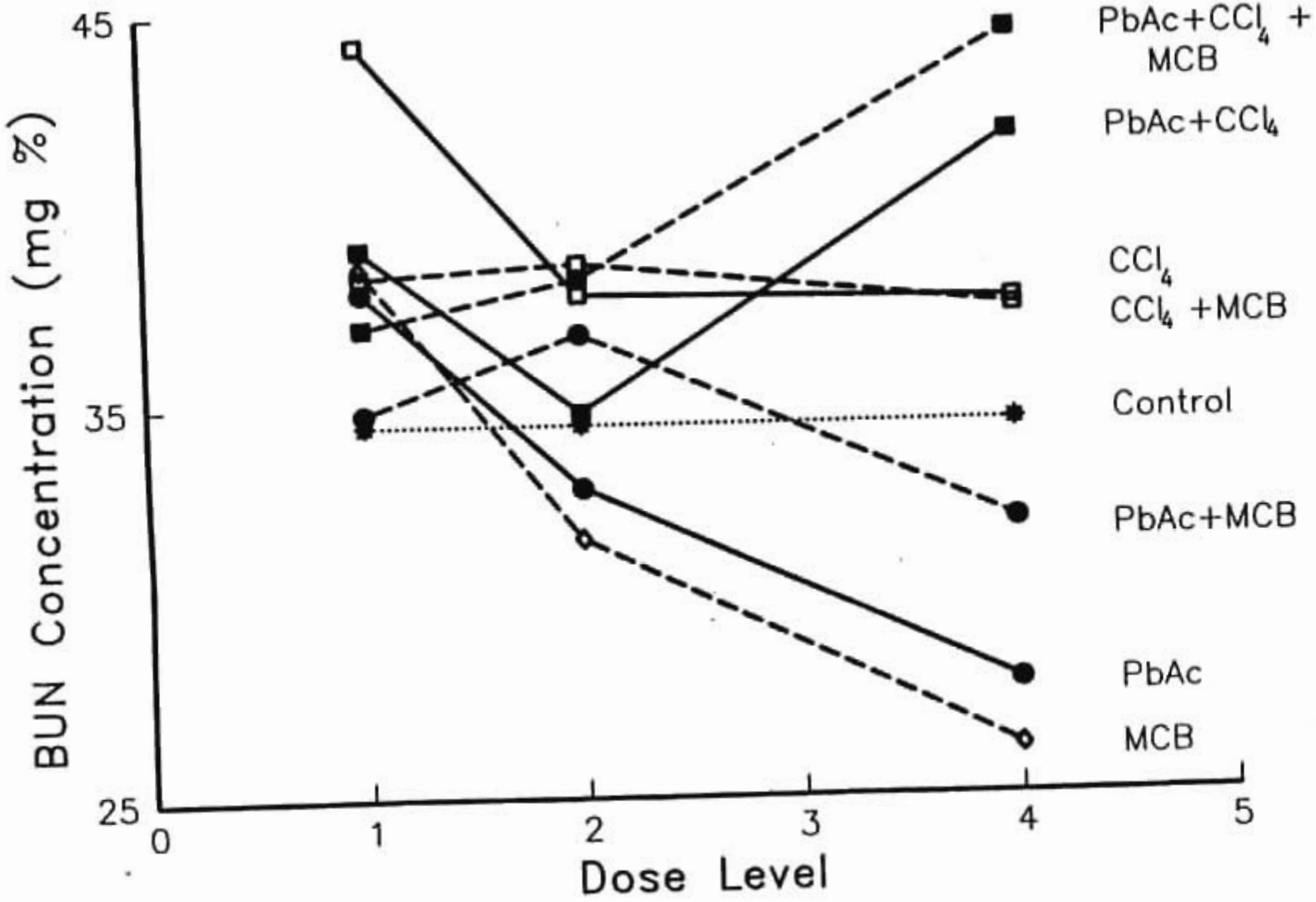
Administration was per os, daily , for 7 days.
For doses and abbreviations, see Figure 1

Figure 6



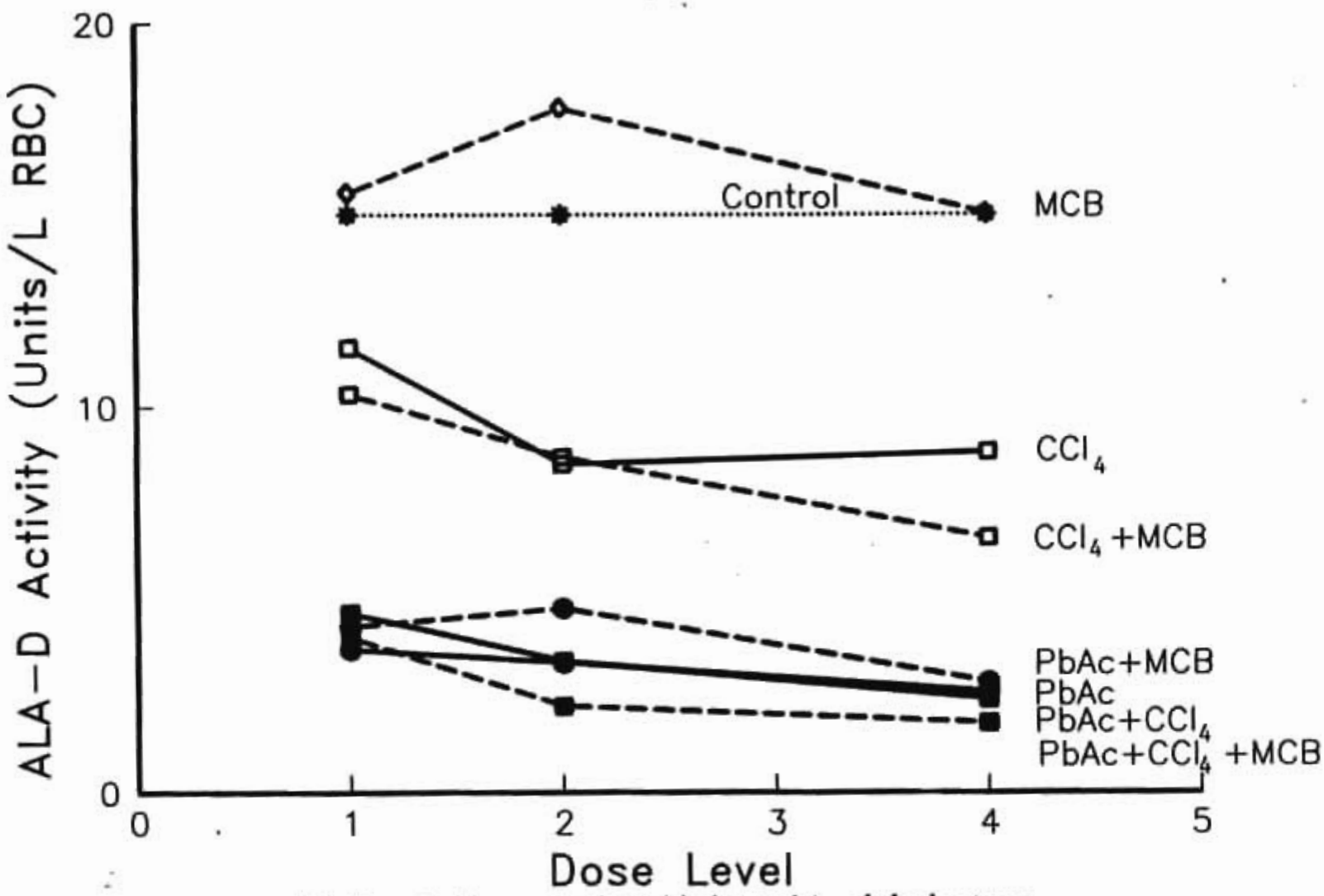
GPT - Glutamic-Pyruvic Transaminase; (L-Alanine Transferase)
SF Units - Sigma Fraenkel Units - one unit will convet 1.0 umole of α-ketoglutarate to L-glutamate per minute at pH 7.6 at 37°, in the presence of L-alanine.
Determination of GPT activity was carried out using a Sigma kit.
Administration was per os, daily , for 7 days.
For doses and abbreviations, see Figure 1

Figure 7



BUN - blood urea nitrogen
BUN was analyzed colorimetrically using a modification of the method of Marsh *et al.*, Clin. Chem. 11, 624-627,1965.
Administration was *per os*, daily, for one week.
For doses and abbreviations, see Figure 1.

Figure 8



ALA-D - Delta-aminolevulinic acid dehydratase.

The assay for ALA-D was carried out by the colorimetric method of Berlin and Schaller (Berlin, A. and Schaller, K.H. (1974) Klin. Chem. Klin. Biochem. 12, 389-390.

For dose levels and abbreviations, see Figure 1

Administration was *per os*, daily, for one week.

Future Work

I. SPECIFIC AIMS FOR THE SECOND YEAR OF THE GRANT.

1. The determination of the potential for selected mixtures to alter the toxicity of known substances.

This aim is two fold, i. e. (1) to detect the potential of synthetic mixtures for such effects and (2) to detect the potential of an environmental mxiture to affect the toxicity of known toxins. We will add components one at a time to the synthetic mixtures, so as to detect interactions as the mixtures become more complex.

2. The identification of the compounds in a mixture which will form adducts with DNA when administered in vivo.

II. Experimental Design.

A. Continuation of Studies with Synthetic Mixtures

The studies initiated this year with synthetic mixtures will be further developed during the second and third years of the grant. The protocols for these experiments will be similar to those for the previous studies in that the same indices of toxicity will be evaluated in male rats exposed to the more complex mixtures. PCBs (Aroclor 1254) will be the next component added to the current mixture which consists of CCl_4, lead acetate and monochlorobenzene (mixture 1). The protocol for the studies with PCBs is shown in Table 8. As in our previous studies, the PCBs will be administered to the rats at three doses, alone and also given in various combinationS with the other three compounds. The PCB component was chosen because PCBs are ubiqoutous in hazardous waste sites and in the environment. Statistical analyses will be those used in the present studies, i.e. the two way analysis of variance.

If unusual toxic responses are found with the PCB administration (alone or as a component of mixtures) we will focus on these responses. PCBs are known to be potent inducers of the hepatic mixed function oxidase enzymes involved in metabolism and thus PCB treatment may cause increased toxicity of MCB or CCl_4, both toxic after metabolic activation. CCl_4, in contrast, decreases the levels of these hepatic enzymes. The response of the animals to the administration of both compounds will give us valuable information on their interactions in vivo. Similar interactions may occur with other compounds in the above mixtures.

B. Investigation of the Effect of Environmental Mixtures on Known Toxins

During the second and third years, we will initially study the effect(s) of environmental mixtures (such as modified leachates) on the toxicity of the known toxins, lead acetate (PbAc) and CCl_4, as well as the carcinogen, dimethylnitrosamine (DMN) (see below). (Our work in year 1 has given us good background data on PbAc and CCl_4). The experiments will utilize three doses of mixture alone, PbAc or CCl_4 alone and mixture plus lead or CCl_4 at the highest doses given to data, i.e. CCl_4 - 5.2 mMoles/kg body weight, PbAc - 2.68 mMoles/kg. All treatments will be given daily for one week by the oral route. In the studies with lead we will focus on

evaluation of renal and hematalogical function. We will measure delta-aminolevulinic acid dehydratase activity, BUN, and kidney/body weight ratios and will also evaluate the damage to susceptible tissues with histological studies. Liver function will be the focal point for studies with CCl_4. Liver/body weight ratios as well as SGPT and alkaline phosphatase activities will be measured and histological evaluation will be carried out for susceptible tissues. In addition, the effect of the environmental mixtures on CCl_4-induced lipid peroxidation will be investigated by measurement of conjugated dienes formed in liver microsomes, using the method of Recknagel and Ghoshal (Lab. Invest.15, 132-148, 1966). We will also measure the hepatic mixed function oxidase (MFO) enzyme components in microsomal fractions, i.e. cytochromes P-450 and b_5 as well as cytochrome C reductase activity by techniques routinely used in our laboratories.

C. Mutagenicity Studies.

Assays for mutagenic metabolites in the urine collected from 2 animals/highest dosed groups will be carried out in addition to the studies described in A and B. The Salmonela typhimurium strains TA 98 and TA 100 obtained from Ames will be used to detect both frame shift and base pair mutagens. Urines will be collected under toluene (in iced containers) while the animals are in metabolic cages. The assay will be carried out using the revised technique of Maron and Ames (Mut. Res. 113,173-215, 1983) following the chromatography of urines samples on an XAD column to remove salts and concenetrate any mutagens. The latter method has been successfully used in our laboratories to detect mutagens in urines of patients receiving chemotherapy.

D. Post-labelling Detection of DNA adducts.

Carcinogens/mutagens bind to DNA in vivo to form adducts with the nucleotide bases; this is the putative initiation step in carcinogenesis. Identification of these adducts will allow the detection of the potential carcinogens/mutagenis in a complex mixture of chemicals. The newly developed method of enzymatic ^{32}P-phosphate post-labeling will be applied to the analysis of adducts formed after administration of both synthetic and environmental mixtures to rats or mice. The method used will be a modification of that of Randerath et al. (Proc. Natl. Acad. Sci, 78, 6126-6129, 1981). The method is based on the fact that the adducts can be labeled with ^{32}P-phosphate and subsequently separated and identified. An important step in the procedure is that of transfer of a labeled phosphate from the gamma position of ATP to the 5' hydroxyl terminus of 3' phosphoryl nucleotides that are derived from modified DNA by appropriate nuclease digestion. Briefly, the compound of interest or the mixture is administered to rodents and 3-4 hours later animals are sacrificed. (The time of sacrifice may be longer with our studies of oral administration). The livers, bits of skin and/or any other tissues of interest are taken for homogenization and digestion. The modified DNA (DNA containing the adduct) is isolated and is digested by two enzymes, micrococcal nuclease and spleen phosphodiesterase, to form 3'-deoxynucleoside monophosphates, i.e. the 5' phosphates are removed. The 3'-nucleotides are then labeled with ^{32}P at the 5 position by the addition of high specific actitivity gamma^{32}P ATP and T4 polynucleotide kinase. A mutant enzyme that lacks the endogenous 3' phosphatase activity is used to label the nucleotides, the products formed being 3',5'-deoxynucleoside diphosphates. The 3'-phosphate group is then

removed by addition of normal T4 polynucleotide kinase which contains 3'-phosphatase activity. The normal four DNA nucleotides are labeled, as are any nucleotides containing modified bases. These compounds are then separated by cation high pressure liquid chromatography or by polyethyleneimine-cellulose thin layer chromatography, the latter in ammonium formate and ammonium sulfate solutions. The unbound bases migrate to form characteristic patterns as do the bases bound to adducts, e.g. methylted bases. Autoradiograms are made of the chromatograms and the resultng autoradiograpahic maps of "normal DNA" and DNA addu__cts contain characteristic spots for specific adducts. Comparison with published "maps" or with those made in our labaoratories from known carcinogens reacting with DNA will permit identification of the compnents reacting with the DNA. Initially we will study the effects of mixtures on adducts formed from DMN, which methylates DNA bases with a well-defined characteristic pattern. This pattern has been studied by Randerath 's group (loc. cit.). A schematic diagram of the steps in the ^{32}P-labelling technique is shown in Table 9, page 27.

In our studies the animals will be given DMN orally at a dose level which must be determined from preliminary studies to result in measurable hepatic aduct formation. (Other tissues may be used later). The animals will be sacrificed and the livers removed and homogenized and the DNA collected from the nuclear fraction by standard techniques of solvent extraction. We will then digest the DNA fraction according to the diagram (Table 9) using snake venom phosphodiesterae and DNAase I for the 5' digestion. The ^{32}P phosphate will be added to te 5' hydroxyl position with polynucleotide kinase. We plan to use thin layer chrormatography to separate the DNA-adduct fractions, and we will then prepare autoradiographs from the thin layer plates. (Our laboratories have previously used this technique for phospholipid separations). For studies of the effects of mixtures, we will administer the DMN and mixtures at the same time and analyze for DNA adducts, as above. We will later expand the method to detect predicted adducts from synthetic mixtures.

Table 8

Experimental Design for Study of the toxicity of lead acetate, monochlorobenzene, and carbon tetrachloride

Group 1 vehicle	Group 10 vehicle	Group 19 vehicle
Group 2 low dose D	Group 11 medium dose D	Group 20 high dose D
Group 3 low dose A + low dose D	Group 12 medium dose A + medium dose D	Group 21 High dose A + High dose D
Group 4 low dose B + low dose D	Group 13 medium dose B + medium dose D	Group 22 high dose B + high dose D
Group 5 low dose C + low dose D	Group 14 medium dose C + medium dose D	Group 23 high dose C + high dose D
Group 6 low dose A + low dose B + low dose D	Group 15 medium dose A + medium dose B + medium dose D	Group 24 high dose A + high dose B + high dose D
Group 7 low dose A + low dose C + low dose D	Group 16 medium dose A + medium dose C + medium dose D	Group 25 high dose A + high dose C + high dose D
Group 8 low dose B + low dose C + low dose D	Group 17 medium dose B + medium dose C + medium dose D	Group 26 high dose B + high dose C + high dose D
Group 9 low doses of A + B + C + D	Group 18 medium doses of A + B + C + D	Group 27 high doses of A + B + C + D

All groups consist of 6 rats each. All compounds and mixtures of compounds are administered per os daily for seven days. The animals are killed and autopsied on the eighth day.

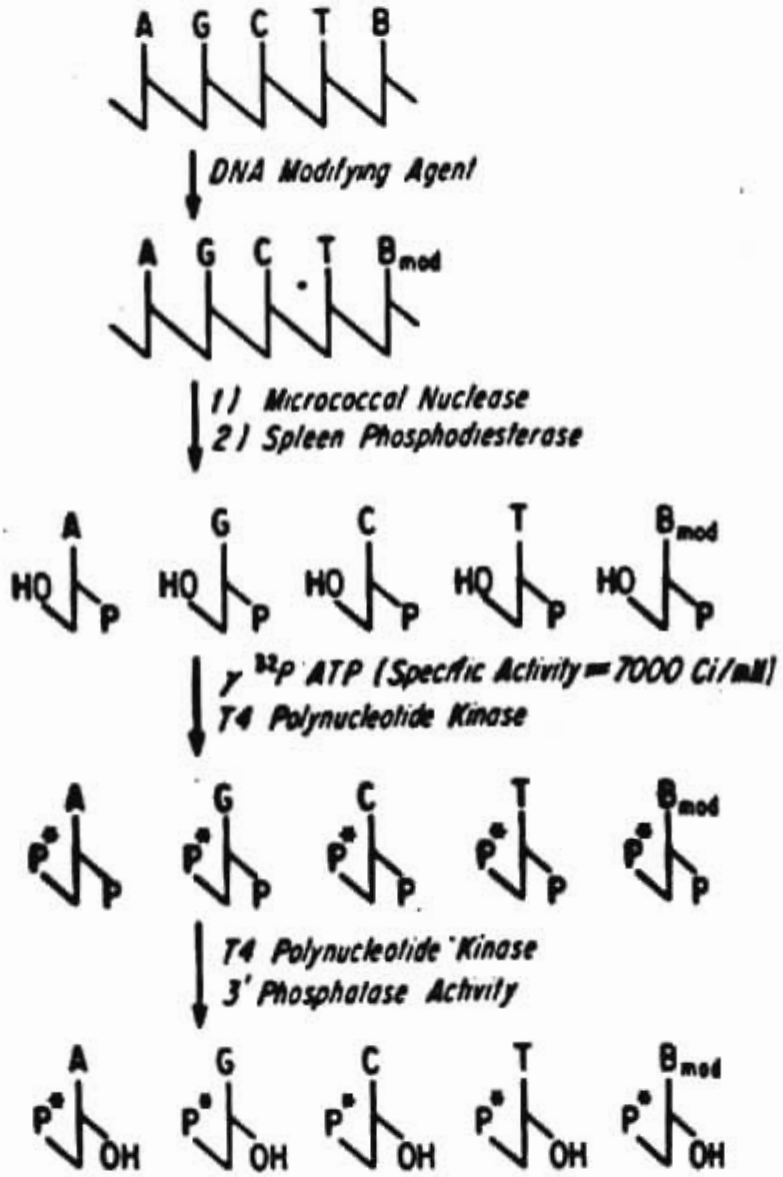
Compound A, lead acetate, will be dosed at 13.7, 27.5, and 55 mg/kg.

Compound B, carbon tetrachloride, will be dosed at 0.125, 0.25, and 0.5 ml/kg.

Compound C, monochlorobenzene, will be dosed at 75, 150, and 300 mg/kg.

Compound D, Aroclor 1254 will be dosed at 12.5, 25, and 50 mg/kg.

Figure 9



Scheme for DNA Post-labeling Technique (From Haseltine, W., Franklin, W. and Lippke, J. Environ. Health Perspec. 48; 29-41, 1983)

BUDGET - HAZARDOUS WASTE GRANT
(Effects of exposure to Multichemical Mixtures)

Personnel	Effort	Salary	Fringe Benefits	
Co-Principal Investigators:				
Charlotte Witmer, Ph.D.	15%	-	-	
Eileen Hayes, Sc. D.	15%	-	-	
Technicians (2)				
Sandra Dutton, M.S.	100%	18,500	4,625	23,125
Linda Evans, B.S.	100%	16,540	4,135	20,675
			total	43,800
Animals and animal care				11,000
Supplies (small pieces of equipment, glassware, chemicals, biochemicals, including radioisotopes)				11,000
Travel (for one of Principal Investigators to attend annual meeting of Society of Toxicology or other major national meeting).				1,100
Other (telephone, xeroxing, publication costs, etc.)				2,200
Direct Costs		(Subtotal)		$67,100
Overhead (5%)				3,355
			Total	$70,455

The above budget represents a 10% increase ~~of 10%~~ in all categories, as the prices of chemicals have increased, and we will be working with animals for a full year during year 2. In year 1 we spent the initial few months validating assays, hiring personnel, etc. Thus the expenses in general were not as high as the expected expenses for the coming year. We also have a high item of radioisotopes, specifically ^{32}P labelled compounds. However, a gain for the grant financially is that a graduate student, funded from departement funds, will spend some time on the ^{32}P labelling method. A medical student spent the summer months on this project this year and was funded from monies available because the first technician to be hired left after a few months to persue his medical career.