COMBINED EFFECTS IN TOXICOLOGY—A RAPID SYSTEMATIC TESTING PROCEDURE: CADMIUM, MERCURY, AND LEAD

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A testing procedure is described for the assessment of the toxicological response (e.g., acute toxicity or mutagenicity) of any combination and number of chemical, physical, and biological agents, with no more effort for a particular combination than for a single agent. The method provides a simple, sensitive, and quantitative index of synergism, antagonism, and additivity, and it has been demonstrated experimentally in rats by determining the acute lethality of combinations of cadmium, mercury, and lead salts. In a combination of two metal salts, the dose of one metal of the pair was fixed at or near the no-effect level while the dose of the second metal was increased until the entire dose-response curve was obtained. To evaluate interactions of the three metals, the previous pair of metals were kept fixed at their combined extrapolated LD1 level, and the third metal was increased. The statistical treatment of the data employed a computer program that did not involve probit transformations, but rather the approximate linear relationship between the fractional response and the logarithm of the dose.

A particular combination could be synergistic, antagonistic, or additive, depending on the relative doses employed. Generally, a combination was synergistic when the most toxic member was present at or near its LD1 dose in the presence of the much less toxic member; the same combination was protective when the least toxic member was present at or near its LD1 dose. The results clarify apparently contradictory reports regarding the biological effects of metal combinations. The application of the testing procedure to combinations of mutagens is described, and an example is cited involving, for a particular bacterial mutagen, a combination of N-methyl-N'-nitro-N-nitrosoguanidine with ethylmethanesulfonate.

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INTRODUCTION

The public, and especially workers in many industries, trades, and professions, are exposed simultaneously to a multiplicity of potentially harmful chemical, physical, and biological agents and stresses. Hence, it is necessary to know the possible adverse effects of two or more agents in order to evaluate potential occupational and environmental hazards and to set permissible levels. Because of the lack of data, the promulgation of acceptable levels of exposure does not, in most cases, realistically take into account the combined effects or interactions of hazardous agents.

The degree to which combined effects over the complete dose-response range are synergistic, additive, or antagonistic with respect to a particular toxicological or genetic manifestation has been virtually unexplored. This is partly because routine experimental testing of combined effects involving three or more agents over the entire dose-response range becomes impractical because of the large numbers of test organisms required and the sometimes excessive time and effort needed for range finding. In even relatively simple and compatible mammalian systems involving only 2 agents, the number of animals needed when a factorial design is employed is about 100, aside from those required for range finding (Marubini and Bonanomi, 1970). In a commonly used method for studying interactions—for instance, of pesticides—compounds are administered in equal fractions of their ED50 or LD50 (Hayes, 1975). Thus, with three compounds the dosages would be a geometric series based on one-third of the ED50 for each. However, this method has certain deficiencies, including a lack of sensitivity, in providing an evaluation of the interactions.

Before the development of the method described here, we initiated a program to investigate systematically the combined effects of combinations of the salts of three nonessential metals, cadmium, mercury, and lead. They were selected because they are among the most toxic and ubiquitous environmental metallic contaminants to which the population is exposed. Further, their concentrations in the human body are relatively high: Cd $\simeq$ 50 mg, Hg $\simeq$ 13 mg, and Pb $\simeq$ 120 mg (Schubert, 1973).

There are a number of publications dealing with the toxicological effects of metal combinations. However, in nearly all cases the experimental findings have been inconclusive and limited because only one or two doses of each metal were tested in combination. It has been suggested that cadmium is a synergistic agent in human lead poisoning (Chisolm and Harrison, 1956; Challop, 1971). The effects of combinations of the salts of mercury with either cadmium or zinc on mammalian embryonic development have been investigated, and it appears that the embryotoxicity of mercury plus cadmium is greater than that of mercury plus zinc (Gale, 1973). The teratogenic effects of a few combinations of cadmium plus lead and cadmium plus zinc indicate that there are both protective and synergistic effects, depending on dosage or end point

(Ferm, 1972). Metals may reduce resistance to infections, as found in mice treated with subclinical doses of lead nitrate and subsequently challenged with *Salmonella typhimurium* (Hemphill et al., 1971). Aerosols of soluble salts of various metals potentiated the response of guinea pigs to sulfur dioxide, apparently by catalyzing its oxidation to sulfuric acid (Amdur and Underhill, 1968).

In the early phases of our experiments, using death as the end point, we found that the usual procedures for testing the combined effects were unsatisfactory and were not sufficiently sensitive and precise for the evaluation of synergistic, antagonistic, and additive interactions. For instance, the administration of an essentially no-response level (LD1) of a mercury salt together with 1/20 of the LD1 of a lead salt killed all of the animals.

We have developed a relatively rapid and systematic technique suitable for the routine testing of combined effects of mixtures of two, three, or more agents. This procedure requires no more animals for a particular combination than for a single agent. It appears to be generally applicable for any number and combination of chemical, biological, and physical agents and stresses, and provides a simple, quantitative, and sensitive index of synergism, antagonism, and additivity for any end point—teratogenic, mutagenic, or cytogenetic.

TEST DESIGN

The procedure for determining the combined effects of three metals was as follows. A dose-response curve was determined for each of the three metals separately. In the investigation described here the acutely lethal doses for the individual metal salts and their combinations were determined. For paired combinations of metals, we maintained one metal at a fixed concentration, namely at or near the essentially no-effect level; this was taken as the LD1 dose obtained by extrapolating a least-squares regression line for the proportion affected versus the logarithm of the dose. Then we increased the dose of the second metal to obtain the usual dose-response curve, using the same number of animals as if this metal were given alone. From the dose-response curve we again obtained an LD1, an LD50, and an extrapolated LD99 for the increased metal in the mixture. We next exchanged the pair of metals so that the increased metal was fixed at its LD1 and the metal that was previously fixed at the LD1 dose was increased. To study the interactions of three metals, we maintained the previously paired metals at their *combined* LD1 and increased the third metal. The extrapolated LD99 dose was determined for future use in the rapid evaluation of the therapeutic effects of different chelating agents compared to mixed-ligand chelates (Schubert, 1972).

We use the convention here that the metals in the numerator were fixed at or near the LD1 and the increased metal is in the denominator.

One can employ any dose near the low end, such as the LD5. When more than one metal is given in the numerator, the first listed was the one kept fixed near its LD1, while the others were increased to obtain the LD1 for the combination. We tested all 15 combinations of cadmium, mercury, and lead: Pb, Hg, Cd, Pb/Hg, Hg/Pb, Pb/Cd, Cd/Pb, Hg/Cd, Cd/Hg, (Pb + Hg)/Cd, (Hg + Pb)/Cd, (Pb + Cd)/Hg, (Cd + Pb)/Hg, (Hg + Cd)/Pb, and (Cd + Hg)Pb.

The results obtained with the combinations were tested for synergism, additivity, or antagonism (protection) by using the dose of the increased agent (expressed in molar units per kilogram for chemicals) needed to attain the LD50 (i.e., LD50 − LD1) and the corresponding value for that agent in the presence of one or more of the other agents. The ratio of these values (actually slopes) provided an index of the degree and nature of the combined effect: synergism > 1, additivity $= 1$, and antagonism < 1. An important finding was that a particular combination could be synergistic or antagonistic, depending on the relative doses employed; this is discussed later.

For the statistical treatment of the data we used a computer program from the program library at Argonne National Laboratory. and an IBM 370/195 computer. The computer calculations provided the LD values for all degrees of response and the standard errors. The slopes, intercepts, and associated standard errors of the least-squares regression lines for each system were determined. Although it is customary to use probit transformations, this proved impractical because of the extreme steepness of the dose-response curves and the need to obtain consistent estimates of LD1 and LD99. Instead, we used the approximately linear relationship between the fractional response and the logarithm of the dose. To obtain the associated standard errors of the extreme LD points, the LD1 and LD99, we used a statistical algorithm described by Rao (1965).

It should be noted that in our statistical treatment of the data we intentionally eliminated a "log-probit model" description of the observed mortality curves because we did not find it worthwhile in this case to make the tacit assumption that toxicity obeys lognormal statistics. In fact, the lognormal model extrapolations gave inconsistent and actually meaningless results at the 1 and 99% levels. Although the dose-response curves are largely linear, their inherently sigmoid shape means that the 1 and 99% values are not necessarily identical to those that might be measured directly. However, we stress that we employed extrapolated but reasonably reproducible LD1 and LD99 values.

MATERIALS AND METHODS

The following metal salts were employed: lead acetate, $Pb(C_2H_3O_2) \cdot 3H_2O$ (formula weight, 379.3); mercuric chloride, $HgCl_2$ (formula weight, 271.5); and cadmium chloride, $CdCl_2 \cdot 2.5H_2O$ (formula weight, 228.3).

They were dissolved in deionized distilled water at concentrations such that no more than 0.3 ml of a single metal salt or 0.6 ml of a combination was injected. The cadmium-mercury combinations formed no precipitate when mixed. However, lead-mercury and lead-cadmium mixtures were not compatible, and it was necessary to inject some solutions separately—for example, lead followed immediately by the mercury and/or cadmium salt.

Male Wistar rats weighing 190–210 g (5–6 wk of age) were used for all the combinations. These are specific pathogen-free rats, cesarean-derived, purchased from Hilltop Lab Animals, Inc., Scottdale, Pa. Since a randomized breeding system was followed, uncertainties because of genetic variability were eliminated. On arrival, the rats weighed 125–150 g (5–6 wk of age) and were equilibrated in our animal quarters until they reached 190–210 g. Animals that did not follow a normal growth curve were discarded. On the average, about 50–60 animals were used for each of the 15 systems, exclusive of those in which the metal doses produced a group mortaility of 0 or 100%. We have also completed toxicity studies on females exposed to the individual metal salts, and the results are reported here.

Rats were injected via the tail vein and only in the morning. The occasional animal that died soon after injection was discarded. At least six animals were injected for each dose of the increased metal. In nearly all cases the doses employed gave a fractional response over a wide range (0.16 –0.8).

Histological effects on the kidneys and, in a few cases, on the testes were observed with mercury and lead salts, singly and in combination. Kidneys were fixed in Bohin's solution for 12 h, washed in 70% alcohol for 6 h, dehydrated in 80, 95, and 100% alcohol, and then cleared and embedded in paraplast. Sections were cut into 5-μm slices on a microtome and then stained with hematoxylin and eosin.

RESULTS AND DISCUSSION

Lethal Doses in Male Rats

Most deaths occurred between 3 and 4 d after injection. Practically no deaths took place at or beyond the d 6. Losses of weight were observed within 24 h and reached a maximum (15–20% of initial weight) at 4–5 d, followed by a recovery to the initial weights at 8–9 d after injection. Gross observation of the internal organs revealed, as expected, that the kidneys were obviously damaged and enlarged.

Typical computer-drawn plots of the fractional response (death) to the dose are illustrated in Fig. 1 for each of the three metal salts. Figure 2 shows the results for the Hg/Pb and Pb/Hg combinations and Fig. 3 those for the (Hg + Pb)/Cd and (Pb + Hg)/Cd combinations. A complete summary of the results is given in Table 1.

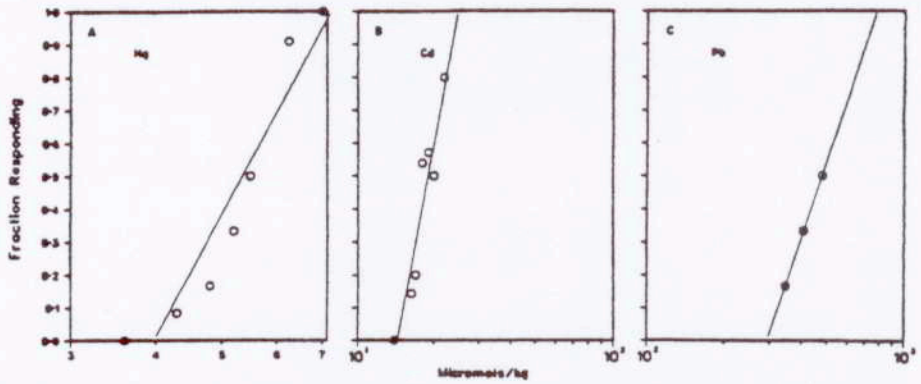

FIGURE 1. Fraction of male Wistar rats killed within 10 d plotted against the logarithm of the dose after a single iv injection of the salts of mercury, cadmium, and lead, respectively. Each point represents a composite or single group of at least 12 animals.

The degree of synergism can be remarkably high. In the Hb/Pb or (Hg + Cd)/Pb combination the acute lethal effect of lead became nearly equivalent to that of mercury. Administration of only 12.4 μmol/kg lead with an LD1 dose of mercury resulted in 50% mortality. This amount of lead is 1/24 of its LD1, or 1/15 of its LD50 in the absence of mercury. It is interesting to note that a combination of the LD1 of each metal is 100% fatal.

The data show that the kind of interaction observed with combinations of metals depends on the relative doses administered. Thus, the mercury-lead combination can be highly synergistic or protective (Table 1). In general, synergism was manifested when the most toxic metal in the

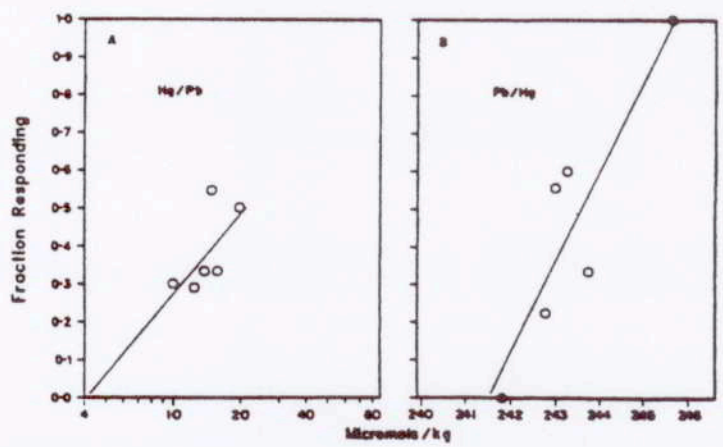

FIGURE 2. As in Fig. 1, but for the administration of a combination of mercury and lead salts. (A) The dose of mercury was fixed while that of lead was incremented. (B) The dose of lead was fixed and that of mercury was incremented.

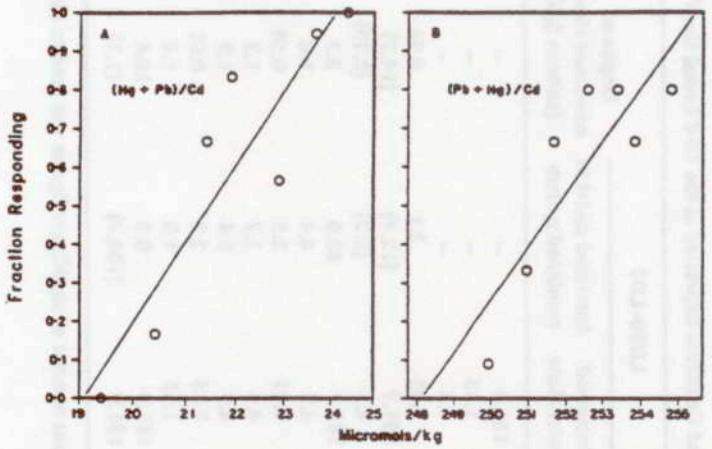

FIGURE 3 As in Fig. 1, but for the administration of a combination of three metal salts.

combination was present in the highest relative concentration—that is, closest to its LD1—in the fixed metal fraction; for example, Hg/Pb, (Hg + Pb)/Cd, and (Hg + Cd)/Pb. When the dose of the least toxic metal was fixed at or near its LD1, the combined effect tended to be antagonistic or, at most, additive, within the experimental uncertainty; for example, Pb/Hg, Pb/Cd, Cd/Hg, and (Pb + Cd)/Hg.

Histology

The kidneys and testes on 4 and 8 d after injection were histologically examined by Dr. Helen L. Lucia; the following 4 groups of rats were used (4 to a group): group I, controls; group II, Pb only, 12.7 μmol/kg (<1/20 of the LD1); group III, Hg only, 4.5 μmol/kg (~LD20); and group IV, Pb and Hg combined, 12.7 μmol/kg Pb and 4.5 μmol/kg Hg. No pathological lesions were found in the testes of any of the animals.

Neither group I nor group II had any pathology in the kidney, and no lead inclusion of the tubular epithelium was found. In the mercury-treated rats, group III, there was massive acute tubular necrosis on d 4. The convoluted tubules, distal and proximal, were massively necrotic. The glomeruli were somewhat congested and contained an occasional polymorph, but were otherwise unremarkable. Some tubules still contained the dead epithelial cells in situ, but in others the epithelium was gone and the bare basement membrane was present. The distal tubules were filled with necrotic debris and dense eosinophilic cast material. The loops of Henle in the medulla were similarly afflicted, although occasional intact epithelium was seen. The interstitium was somewhat edematous, and a few small collections of round cells were seen. The vasculature was unremarkable, as was the urinary epithelium of the calyces.

By d 8 there was significant recovery from the renal insult. The necrotic epithelium, the debris, and the cast material were cleared from

TABLE 1. Acute Lethality in Rats after Intravenous Injection of Combination of the Salts of Lead, Mercury, and Cadmium Expressed as the 10-d Lethal Dose[a]

Metal or combination	Fixed metal doses (μmol/kg)		Total fixed metals (μmol/kg)	(LD1)[b] (μmol/kg)	LD50 (μmol/kg)	(LD99)[b] (μmol/kg)	Slope	LD50–LD1		Degree of combined effect[c] (column 8/9)
								Increased metal alone	Increased metal in combined system	
Pb	–	–	–	295.7 ± 0.4	477.6 ± 0.6	771 ± 1	2.35 ± 0.02	181.9	–	–
Hg	–	–	–	4.00 ± 0.12	5.35 ± 0.16	7.17 ± 0.22	3.87 ± 0.57	1.35	–	–
Cd	–	–	–	14.5 ± 0.3	19.0 ± 0.4	24.8 ± 0.5	4.20 ± 0.67	4.5	–	–
Pb/Hg	240.0	–	–	241.7 ± 0.3	243.8 ± 0.3	246.0 ± 0.3	129.4 ± 33.4	1.35	2.1	0.64
Hg/Pb	4.8	–	–	(5.77 ± 0.65)	(18.15 ± 2.04)	(57.1 ± 6.4)	(0.984 ± 0.542)	181.9	(12.4)	(14.7)
Pb/Cd	240.0	–	–	(246.7 ± 1.4)	(252.9 ± 1.4)	(259 ± 1)	(45.5 ± 29.9)	4.5	(6.2)	(0.73)
Cd/Pb	14.0	–	–	203.3 ± 10.7	283.9 ± 15.0	396 ± 20	3.38 ± 1.47	181.9	80.6	2.3
Hg/Cd	4.8	–	–	11.2 ± 0.3	15.6 ± 0.4	21.5 ± 0.5	3.48 ± 0.50	4.5	4.4	1.0
Cd/Hg	14.0	–	–	15.0 ± 0.5	17.5 ± 0.5	20.5 ± 0.6	7.30 ± 2.64	1.35	2.5	0.54
(Pb + Hg)/Cd	240.0	2.0	242	248.2 ± 0.5	251.9 ± 0.5	255.6 ± 0.5	77.3 ± 21.8	4.5	3.7	1.2
(Hg + Pb)/Cd	4.8	4.6	9.4	19.1 ± 0.3	21.5 ± 0.6	24.1 ± 0.4	9.80 ± 2.05	4.5	2.4	1.9
(Pb + Cd)/Hg	240.0	12.0	252	251.06 ± 0.04	253.28 ± 0.04	255.50 ± 0.04	129.3 ± 6.9	1.35	2.2	0.61
(Cd + Pb)/Hg	14.0	191	205	206.2 ± 0.2	207.2 ± 0.2	208.2 ± 0.2	243.3 ± 82.1	1.35	1.0	1.4
(Hg + Cd)/Pb	4.8	5.2	10	9.18 ± 0.59	19.08 ± 1.23	39.7 ± 2.6	1.543 ± 0.518	181.9	9.9	18.4
(Cd + Hg)/Pb	14.0	2.0	16	(68.4 ± 7.0)	(173.1 ± 17.8)	(438 ± 45)	(1.21 ± 2.00)	181.9	(104.7)	(1.7)

[a]Values are given as the mean ± SE. Values in parentheses are not statistically significant at $p < 0.05$. A sufficient number of significant figures was used to obtain nonzero standard errors.

[b]Obtained by extrapolation of least-squares regression line of proportion affected versus log dose.

[c]Index of combined effect: additivity = 1; protection < 1; synergism > 1.

the tubules. The tubules were quite dilated and were lined by a flat, basophilic epithelium, which showed some mitotic figures. There was more round cell infiltration of the interstitium. The glomeruli, vasculature, and urinary epithelium were unremarkable.

In group IV, which received both lead and mercury, the kidneys showed the same lesions as those of group III (mercury alone). There was no significant increase in the severity of the already massive lesion.

It should be noted that the ability of the rat to survive and repair the mercury-induced lesion is remarkable. The lesion was classic and agrees with the descriptions of human kidneys after the ingestion of 0.9–1.2 g $HgCl_2$ (Heptenstall, 1966).

Since the dose of mercury given before the histological examinations discussed above produced such severe effects in the kidney, further histological examinations of the kidney were made after a lower dose of mercury and a higher dose of lead, as follows: group I, controls; group II, Hg alone, 1.7 μmol/kg (< LD1); group III, Pb alone, 296 μmol/kg (LD1); and group IV, (Pb + Hg), 296 μmol/kg Pb and 1.7 μmol/kg Hg.

There were no pathological findings at d 4 and 8 in group II, which received only mercury. In group III (lead only) kidney changes were minimal. An increased number of sloughed necrotic tubular epithelial cells could be found in the lumen of the straight tubules. In addition, the number of mitotic figures in tubular epithelial cells increased from an average of 0.2 to 1 per high-power field. These changes were clearly seen at d 4 but were still present at d 8. No other pathological changes were noted.

In group IV (mercury plus lead) moderate acute tubular necrosis was present. The lesion was similar to that described in the first test groups, in which the distal proximal tubules and the proximal distal tubules were most affected. Changes consisted of necrosis and sloughing of tubular epithelial cells, flattening of remaining and regenerating epithelium, tubular dilation, and eosinophilic tubular casts. On d 4 the changes were acute, but by d 8 the regenerative process was under way and a few knots of polymorphonuclear leukocytes were present.

These limited histological results for group IV provided evidence of a synergistic effect with Hg/Pb involving damage to the kidney. We plan to extend these studies to other combinations of doses, including cadmium.

There have been numerous studies of the histological effects in rodents of acute and chronic doses of mercury (Ware et al., 1975; Haber and Jennings, 1964; Friberg and Nordberg, 1972; Clarkson, 1972; MacGregor and Clarkson, 1974), cadmium (Friberg et al., 1974), and lead (Goyer and Moore, 1974).

The female rat was more resistant to kidney damage from lead and mercury salts (Table 2). Sex differences in the kidney response to various agents were observed by Haber and Jennings (1964). They first reported sex differences in the effects of a single iv dose of mercuric chloride (2

TABLE 2. Acute Lethality in Male and Female Rats of Lead, Mercury, and Cadmium Salts after a Single Intravenous Injection[a]

Metal	Male[b]				Female			
	LD1[c]	LD50	LD99[c]	Slope	LD1[c]	LD50	LD99[c]	Slope
Pb	295.7 ± 0.4	477.6 ± 0.6	771 ± 1	2.35 ± 0.02	360 ± 16	543 ± 25	819 ± 37	2.74 ± 0.55
Hg	4.00 ± 0.12	5.35 ± 0.16	7.17 ± 0.22	3.87 ± 0.57	4.31 ± 0.08	5.89 ± 0.10	8.05 ± 0.14	3.62 ± 0.37
Cd	14.5 ± 0.3	19.0 ± 0.4	24.8 ± 0.5	4.20 ± 0.67	16.3 ± 0.3	20.1 ± 0.4	24.8 ± 0.5	5.39 ± 1.04

[a] See Table 1 and text for details.
[b] Data from Table 1.
[c] Extrapolated values.

μmol/kg) in the renal tubules of Sprague-Dawley rats. For a particular dose, the male was more susceptible to proximal tubular necrosis produced by minimally toxic doses of mercury. It is reasonable to conclude that the reduced acute toxicity of mercury as well as lead in our female Wistar rats reflected less severe renal damage and more efficient regenerative processes compared with males.

CONCLUDING COMMENTS

The observed combined effects can be rationalized in part by a simplistic model. Assuming that the metals occupy the same or neighboring critical receptor sites in the target tissue or cell membrane, most likely SH groups (Goyer and Moore, 1974; MacGregor and Clarkson, 1974; Friberg et al., 1974), then occupation of these sites by the less toxic metal partially blocks the deposition of the more toxic metal, resulting in a protective effect.

This model is in line with the results of Garber and Wei (1972), who found that pretreatment of mice with lead nitrate (10–20 mg/kg or 30–60 μmol/kg) protected them against the acutely lethal effects of 600 μmol/kg Pb. The protection was transient, the optimal time of pretreatment being 4 d before the challenge dose. Of further interest was the observation that pretreatment with lead reduced the mortality of mice subsequently challenged with mercuric chloride, but not cadmium chloride. These findings agree with ours (Table 1).

When the mercury dose was near the LD1 dose, far less of the less toxic metal, lead, was needed to saturate or exceed a critical level of the remaining critical sites, since less of the increased agent was needed to reach the end point; thus a synergistic effect was produced. In a sense, we are dealing with mass action phenomena in that the factors determining the combined effect include (1) the intrinsic affinity of the individual metals for the critical sites, and (2) the relative concentrations and distribution of the metals within the target organ sites.

The crude model described above can be formulated in terms of the effective dose (ED) required to reach a particular level of a particular biological end point. Using the ED50, for example, the fractional approach α to the end point can be written:

$$\alpha = \frac{ED50 - < ED50}{ED50}$$

In the present investigation, α varies with the amount of the metal kept at a fixed dose. Thus, α approaches zero as the fixed dose approaches the LD50, and α approaches unity as the fixed dose is decreased. Consequently, the smaller the value of α, the greater the likelihood that the additional stress of exposure will require smaller doses of the same or

other metals to produce mortality. For example, in the Hg/Pb system the fixed dose of mercury was reduced from 4.8 to 3.8 and 2.4 μmol/kg, giving values of α for mercury of 0.10, 0.29, and 0.55, respectively, calculated from the measured value of LD50 (Table 1). When the fixed dose of mercury was 2.4 μmol/kg ($\alpha = 0.55$), the LD50 for the mercury-lead combination was essentially the same as that of lead in the absence of mercury (Fig. 4). However, as the fixed doses of mercury increased, the LD50 of the combination decreased, rapidly and linearly approaching the LD50 for mercury in the absence of lead. In other words, less and less of the increased metal, lead, was needed to kill the animals—a synergistic effect was manifested.

As noted previously, in the Pb/Hg system a small degree of protection was observed when the fixed dose of lead was 240 μmol/kg, corresponding to an α of 0.50. It would be interesting to determine whether an increase in the fixed dose of lead to give the α that was synergistic in the Hg/Pb system ($\alpha = 0.2$, or 382 μmol/kg) would lead to synergism instead of antagonism.

Clearly, the considerations above serve mainly as a basis for the design of systematic experimental investigations of combined effects relative to α values or some function or combination of α values. Factors requiring study include differential tissue distributions, relative sites of action, and histological examinations.

The assessment of interactions utilizing mortality as the biological end point is a gross one. However, the combined effects procedures described

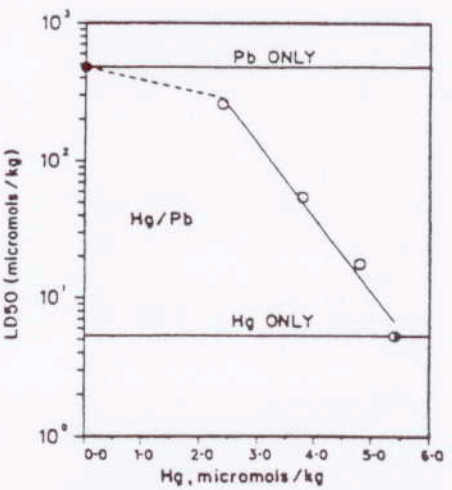

FIGURE 4. Effect of different fixed doses of mercury salts on the LD50 of an Hg/Pb combination. For each fixed dose of mercury, the lead dose was increased. Otherwise the procedure was as described in Fig. 1.

here can be used for more sensitive and subtle biological or biochemical end points. For example, one of the earliest evidences of an adverse effect of lead was the inhibition of δ-aminolevulinic acid dehydrogenase (ALAD) in red blood cells caused by interference with heme biosynthesis (Hernberg et al., 1970). Using the procedures described here, we can investigate the effects of mercury and cadmium, for example, on lead-induced inhibition of ALAD activity. The nature and degree of interaction would be assessed as described above, namely from the dose of lead alone required to decrease ALAD activity from, say, 5 to 50%, compared with the dose required in the presence of mercury and cadmium (see columns 8, 9, and 10 in Table 1).

Other sublethal or chronic indices of interaction of these metals can be employed, including target organ functions, immune responses, or histology. Further, one can determine the degree of interaction involving subpopulations with differnt susceptibilities, such as age groups. The combined effects procedure is limited only by the ability to quantitate the desired end point.

Combined Mutagens

We (J. M. Gentile and J. Schubert, in preparation) have applied the testing procedure described here to the Ames mutagenicity test (Ames et al., 1975) with histidine-deficient mutants of *S. typhimurium*. As an index of response we employed the increase in mutation frequency (MF) over the spontaneous mutation rate. We generally maintained the fixed concentration of mutagens at a nearly no-effect level—namely a concentration (MF_{2x}) that doubles the spontaneous mutation frequency. The concentration of the other mutagen was increased until the MF reached a maximum. To determine the extent of synergism, antagonism, or additivity, we determined the combination of mutagens needed to reach half the maximum reading, from which we obtained values of ($MF_{max/2} - MF_{2x}$).

In one combined system we used bacterial tester strain TA100 for the detection of mutagens causing base-pair substitutions. We tested the combination of a very strong mutagen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG), with a relatively weak mutagen, ethylmethanesulfonate (EMS). The latter barely doubled the spontaneous mutation frequency in our system, even at concentrations of 0.5–1.0 *M*. However, when we used a fixed dose of NG at the MF_{2x} level (10^{-4} *M*), a striking increase in mutation frequency was observed with EMS concentrations in the range 10^{-3}–10^{-4} *M*. In fact, the NG/EMS combination proved lethal at concentrations of EMS roughly greater than 10^{-3} *M*. The synergistic effect was observed, however, only when NG was first added to the top agar, followed by EMS. When the chemicals were mixed by sonication before addition, no synergism occurred.

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