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MASTER

MIXED LIGAND CHELATE THERAPY
FOR PLUTONIUM AND
TOXIC METALS FROM ENERGY POWER PRODUCTION

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ABSTRACT

It is shown that a mixed ligand chelate (MLC) is superior to that of the primary chelate alone for the decorporation of monomeric plutonium. Thus, EDTA plus Tiron reduces the level of Pu in the skeleton about 40% less than that of EDTA alone when treatment was begun two hours post ^{239}Pu . However, DTPA alone was still superior to EDTA/Tiron under the given experimental conditions. However, this appears to be due to the rapid metabolic destruction of secondary chelants such as Tiron and similar bi-dentate derivatives of catechol and salicylates. Investigations are under way to improve such MLC's by the use of metabolic inhibitors to increase the plasma concentrations and residence times of the secondary chelants. In addition, MLC's consisting of metabolically stable chelants are undergoing testing. Therapy of cadmium poisoned mice by single and mixed chelants is also under study. A molar ratio of EDTA to Cd of 1.6 reduces the mortality by half of female mice given 5 minutes (i.p.) post supralethal doses of CdCl_2 , while a molar ratio of 4.7 for 2,3-dimercaptopropanol-1-sulfonate (DMPS) is required. No protection is observed with penicillamine (PA) or salicylates (SA) alone. However, combinations of EDTA with both PA and DMPS or SA and DMPS afford protection under conditions (30 minutes post Cd) where the individual chelants are ineffective. Automated potentiometric titrations are utilized to detect MLC formation and to evaluate their stability. The titration data are used as a screening procedure to ascertain whether a given chelant combination is worth testing *in vivo*.

I. INTRODUCTION

During the past nine months we have made improvements in our analytical and screening procedures. These permit us to complete experiments on the decorporation of plutonium and toxic metals in a fraction of the time previously required and with improved accuracy.

We have introduced rapid, computerized potentiometric titration as a rapid and reliable method for diagnosing whether mixed ligand chelate (MLC) formation has taken place, and, if so, the stability of the MLC relative to the usual 1:1 chelate, e.g., of the metal with EDTA or DTPA.¹ Therefore, those combinations of chelants which do not form significantly stable MLC's are eliminated from biological testing. We have also refined our biological-toxicological procedures. The net result of these and other modifications means that during the coming year we will be able to reach definite conclusions regarding the effectiveness of MLC formation relative to that of DTPA for the decorporation of plutonium, particularly the insoluble states--polymers and oxides as well as in the treatment of toxicity by metals such as cadmium, mercury, and chromium.

II. RESULTS

1. Analytical Procedures

a) Rapid wet ashing

Together with Prof. Klein and B. Ackerman, a student research assistant, we applied an ashing procedure first described by Seidel and Volf for acid digestion of tissues in preparation for determining ²³⁹Pu by ligand scintillation spectrometry (Int. J. Appl. Radiat. Isotopes 23, 1-4, 1972). The oxidizing mixture consists of a 1:1 mixture of 70% perchloric acid and 30% H₂O₂. Briefly, the oxidizing mixture is added to the scintillation vial containing the tissue, the mixture is allowed to stand overnight, and then heated for 2½ hours at 70°C. After cooling the scintillation solution is added and the vial is ready to be counted.

We found that certain modifications in technique had to be made for liver samples. Further details are given in the accompanying renewal proposal.

¹Abbreviations for various chelants mentioned in this report are BAL-2,3-dimercaptopropanol; CAT-catechol; DCTA-1,2-diaminocyclohexanetetraacetic acid; DFOA-desferrioxamine-β-methane sulfonate; 2,3-DHB-2,3-dihydroxybenzoic acid; dithiocarb-sodium diethyldithiocarbamate; DMPS-2,3-dimercaptopropanol-1-sulfonate; DND-1,8-dihydroxynaphthalene-3,6-disulfonate; DTPA-diethylenetriaminepentaacetic acid; EDTA-ethylene-diaminetetraacetic acid; HEDTA-hydroxyethylenediaminetetraacetic acid; NTA-nitrilotriacetic acid; PA-penicillamine; PCA-protocatechuic acid; RES-resorcylic acid; SA-salicylic acid; SHA-salicylhydroxamic acid; Tiron-1,2-dihydroxybenzene-3,5-disulfonate; TRIEN-triethylenetetramine, dihydrochloride.

The important advantages over our earlier HNO_3 ashing procedure are time--analyses are complete within $2\frac{1}{2}$ days compared with weeks; and the possibilities of cross contamination are considerably reduced. All this means that when multiple treatments are carried out that we can modify the treatment schedule since the decorporative effectiveness can be determined before subsequent treatments are given. Even more important is the fact that we can now evaluate the effectiveness of many more chelant systems for Pu removal.

b) Scintillation counting of ^{239}Pu

We have also carried out careful calibration of the scintillation counter setting. Results obtained on samples ashed as described above are highly satisfactory from both the accuracy and precision of the results.

2. Detection of MLC Formation by Potentiometric Titration

Potentially hundreds of chelant combinations may form MLC's having stabilities for Pu binding much greater than that of DTPA, our reference chelant. However, to carry out *in vivo* decorporation experiments with so many possibilities is not practical. For this reason we are carrying out titrations of metal-chelant combinations. This procedure enables us to detect significant MLC formation and to obtain structure-activity relationships which enable us to choose more reliably those combinations of chelants most likely to form MLC's *in vivo* which might be more effective than DTPA.

In collaboration with Prof. M. Seymour, we are employing an automated titration assembly, details of which are described in the renewal proposal. The titration operation is placed under control of a microcomputer which improves the precision of the titration and eliminates the necessity for manual recording. The titration data are stored on magnetic tape for use later in the calculation of formation constants.

Briefly, the principle employed for detecting MLC formation is simple, straightforward, and reliable. Consider a system consisting of Th^{4+} , a Pu^{4+} stand-in, EDTA, and catechol (CAT). The EDTA has 6 sites to combine with Th and 4 protons which we designate H_4EDTA while catechol has 2 tightly bound protons, H_2CAT . The first 2 protons of EDTA are loosely bound while the last 2 do not become titratable until about pH 7 and 10 respectively. However, when chelate formation with EDTA takes place, even the 2 tighter bound protons are released and titratable at pH's slightly above 3. In the case of catechol, neither proton is released with or without Th^{4+} present. However, if MLC formation takes place then all of the protons from EDTA and catechol become displaced and titratable at lower pH's. Obviously, the MLC formation must not only be significant as determined from the ease with which the tightly held protons are displaced but also the MLC formation must take place at a pH low enough for effective *in vivo* activity.

If little or no MLC formation takes place, the titration curves for Th-EDTA will be identical or nearly so with that of Th-EDTA-S where S is the secondary chelant.

Consider the reaction of Th^{4+} with $\text{Na}_2\text{H}_2\text{EDTA}$.

Hence a total of 6 protons will be released if MLC formation takes place.

In Figure 1 is shown typical titration curves of Th with EDTA and DTPA in the presence and absence of Tiron, Catechol, and salicylic acid (SA). Note that while Tiron and CAT form strong MLC's with EDTA or DTPA the SA does not form a significant MLC with DTPA but does with EDTA, albeit weaker by far than Tiron or CAT. In general, catechol derivatives form particularly strong MLC's, not only with Th, but with divalent transition elements such as Cd^{2+} and Cu^{2+} .

Figure 2 shows that DMPS forms an MLC with EDTA. The reaction; therefore must be as follows:

Both DTPA and EDTA form strong MLC's with Tiron and CAT but the MLC of EDTA with these bi-dentate compounds are fully formed at lower pH's than that of DTPA.

We are now calibrating all our chelants. We are now carrying out systematic investigations of the complexing reactions involving a series of primary chelants with secondary chelants. I have made arrangements for the quantitative calculations of the formation constants to be made in collaboration with Prof. Daniel Leussing of the Ohio State University. Prof. Leussing has a computer program for carrying out these calculations. Further details are discussed in the accompanying renewal proposal.

3. Chelation Therapy of Cadmium Poisoning

In order to determine whether one obtains a synergistic protective effect against acutely lethal doses of Cd because of MLC formation, it is necessary to administer non-protective doses of the single

FIGURE 1

Titration Curves in 0.1 M KNO₃ Showing MLC Formation (25⁰C)

$[\text{Th(IV)}] = [\text{Na}_2\text{H}_2\text{EDTA}] = [\text{Na}_3\text{H}_2\text{DTPA}] = [\text{Na}_2\text{H}_2\text{Tiron}] = 2 \times 10^{-3} \text{ M}$

pK's: Tiron = 7.6 12.6

Na₂H₂EDTA = 6.1 10.2

Na₃H₂DTPA = 8.5 10.5

- Notes:
1. The MLC curves for catechol are similar to Tiron
 2. In the presence of salicylate little difference is observed between ThDTPA and ThDTPA/SA while about a 50 microliter displacement is seen with ThEDTA
 3. Upon MLC formation all four H⁺ are displaced at relatively low pH

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FIGURE 2
MIXED LIGAND CHELATE FORMATION

POTENTIOMETRIC TITRATION OF Cd^{2+} by EDTA

AND IMPs (Each conc'n = 0.03 mmols)

All concentrations = 2×10^{-3} M each.

A = DMPS ; B = Na_2EDTA ; C = Cd/DMPS

$$D = \text{Cd/EDTA} ; E = \text{Cd/EDTA/DMPS}$$
$$pK - \text{CdEDTA} = 16.4 ; pK_{\text{H}} = 2.0; 2.7; 6.2; 10.2$$

CdDMPs = 16.2 $pK_{11} = 8.8 ; 11.2$

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chelants. Consequently we are systematically obtaining dose-response data in our HA ICR female mice (20-25 grams) given supralethal i.p. doses of $\text{CdCl}_2 \cdot 2\frac{1}{2} \text{H}_2\text{O}$ followed at a fixed time post Cd with varying dose levels of a given chelant.

The following summary of toxicity data is incomplete since the statistical analyses will not be completed until additional points are observed but it shows the nature of the investigation in progress. It does demonstrate that several MLC's show promise, however.

a) Acute toxicity of cadmium chloride

The mice were given single i.p. injections of varying doses of $\text{CdCl}_2 \cdot 2\frac{1}{2} \text{H}_2\text{O}$. Death from kidney damage occurred within 1-3 days depending on the Cd doses. The LD 50/10 days is 0.029 mmols/kg (Fig. 3). The no-effect dose is about 0.02 mmols/kg and the minimum LD100 is about 0.04 mmols/kg. Studies on the therapeutic effect of chelants are generally carried out by us with doses of 0.075 mmols/kg of Cd, a supralethal dose which kills most of the mice within 24 hours, and the balance within 48 hours post Cd.

b) Chelation therapy of acute Cd poisoning

Two different LD100 doses of Cd were employed - 0.055 mmols/kg and 0.075 mmols/kg. When treatment was begun 5 minutes post Cd the lethality was reduced by one-half by CaNa_2EDTA when the molar ratio in mmols/kg of $\text{CaNa}_2\text{EDTA}/\text{Cd} = 1.6$ and with DMP the corresponding molar ratio was 4.7 (see Fig. 4). With 0.075 mmols/kg of Cd, a dose of 0.12 mmols/kg of CaNa_2EDTA and of 0.35 mmols/kg of DMPS reduced the mortality by one half. Even 1.0 mmols/kg of SA had no effect on the Cd toxicity. Doses of PA as high as 0.6 mmol/kg were ineffective.

When treatment was delayed 15 minutes post Cd no protective effect of CaNa_2EDTA was obtained at a dose of 0.15 mmol/kg while 0.6 mmol/kg of DMPS reduced mortality to 40%. After a 30 minute delay, 0.4 mmol/kg of DMPS was ineffective while 1.0 mmol/kg gave complete protection. At a 60 minute delay, even 0.5 mmol/kg of EDTA was ineffective. These studies are obviously incomplete at this stage.

c) MLC therapy of Cd poisoning

Until the individual chelant protective effects as a function of dose and time post Cd are complete, we cannot carry out extensive studies of the effects of combinations (see the accompanying renewal proposal). Certain preliminary findings are, however, summarized here (Table 1). The results suggest that synergistic effects do occur, especially when EDTA is used together with PA and DMPS. Other systems are under investigation by potentiometric titration and *in vivo* testing. These include DTPA, SHA, and resorcylic acid.

d) Mercury poisoning

We have begun investigations on mice given lethal doses of mercuric chloride and then treated with chelants. As yet we have not carried out titration measurements. We have, however, utilized

FIGURE 3

Acute lethality to $\text{CdCl}_2 \cdot 2\frac{1}{2} \text{H}_2\text{O}$
of female HA ICR mice (20-25 grams)
Mice given single i.p. injection
Death occurred within 10 days

FIGURE 4

Effect of Dose of CaNa_2EDTA and
DMPS on the Lethality of LD100
Doses of $\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$ in Female
Mice (20-25 grams)

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TABLE 1
Preliminary Results on Survival of
HA ICR Female Mice
Given Single and Mixed Chelants
Post 0.075 mmol/kg CdCl₂

Time Treatment Given Post Cd	System ^a	Doses (mmols/kg)	Mortality %
--	Cd only	0.075	100
5 min	EDTA/PA	0.15/0.6	80
"	EDTA/DMPS	0.1/0.4	80
"	EDTA/SA	0.1/0.4	20
15 min	EDTA/SA	0.1/0.4	100
"	EDTA/SA	0.15/0.6	80
"	EDTA/DMPS	0.15/0.6	0
"	DMPS	0.6	40
"	EDTA	0.15	100
30 min	EDTA/DMPS	0.1/0.4	100
	EDTA/SA	0.1/0.4	100
	EDTA/PA/SA	0.15/0.3/0.3	100
	EDTA/PA/DMPS	0.15/0.3/0.3	40
	EDTA/SA/DMPS	0.15/0.3/0.3	60
	DMPS	0.4	100
	DMPS	1.0	0

^aThe EDTA was administered (i.p.) as the CaNa₂EDTA salt.

chelants which are expected to complex Hg^{2+} because of their structures and donor atoms.

The dose-response of the mice to HgCl_2 is shown in the computer drawn plot (Fig. 5). The lethal doses using 50 mice were obtained from our computer program (see Schubert, J., Riley, J.A., and Tyler, S.A., J. Toxicol. & Env. Health, 4, 763-776, 1978) and are summarized in Table 2.

TABLE 2

Mortality after Ten Days in Female Mice (20-25 grams)

Given HgCl_2 (i.v.)

	Dose $\pm$ S.E. ($\mu\text{mol/s/kg}$)
LD1	8.90 $\pm$ 0.43
LD50	17.2 $\pm$ 0.8
LD99	33.3 $\pm$ 1.6

When a single treatment with a chelating agent was given 10 minutes post Hg, all the mice survived when given 1.0 mmol/kg of DMPS or DMSA but all died when given 1.0 mmol/kg of Tiron or TRIEN. These preliminary studies will be resumed on a systematic basis when the Cd investigations are completed.

e) Pu decorporation

Potentiometric titrations of Th(IV)-DTPA-SA systems, using Th(IV) as a stand-in for Pu(IV), showed no or weak MLC formation. Therefore, I had to assume that previous results which indicated that DTPA/SA was far superior to DTPA alone for Pu decorporation were incorrect. Upon rerunning the experiments myself with new assistants and using the new analytical techniques, I found, within experimental error, no enhanced effect of SA upon DTPA decorporation. These experiments were carried out by beginning treatment 3 days post Pu and continuing treatment twice weekly for a total of 4 weeks, or 8 treatments in all.

As a further check, I ran experiments using Argonne National Laboratory (ANL) mice since ANL reported (Dr. M.H. Bhattacharyya) to me that they found no enhanced effects of SA on DTPA decorporation. Within experimental error no enhanced effect of SA on DTPA decorporation was observed, in agreement with unpublished findings from Argonne. With delayed treatment neither did we find any synergism between EDTA and Tiron.

FIGURE 5

Mortality of HgCl_2 in Female Mice (20-25 grams). Mercury given i.v. The curve is computer drawn (Schubert et al, Toxicol. & Env. Health 4, 763--

Since the secondary chelants employed are rapidly metabolized, it is preferable, at this stage, to test MLC effects when the chelants are administered shortly after Pu. Accordingly, we are now carrying out such experiments.

As of this writing we carried out experiments in which EDTA or DTPA with three different secondary chelants were administered twice daily for two days beginning two hours post Pu. From the chemical standpoint, EDTA/Tiron must be more effective than EDTA alone. The results, described in Table 3, show that indeed EDTA/Tiron was significantly superior to EDTA alone, reducing the femur and liver levels of Pu about 40% and 25% respectively below that of EDTA. However, EDTA/Tiron was not as effective as that of DTPA alone under the same conditions. That of DTPA/Tiron however, appeared to be slightly (~12%) more effective than DTPA alone.

These results demonstrate that, in principle, MLC formation is capable of enhancing the effects obtained with a primary chelant. Since EDTA/Tiron forms a much stronger chelate than DTPA, the greater effectiveness of the latter indicates that Tiron is so rapidly destroyed by metabolic transformation, that the MLC is prevented from manifesting its true potential. This is shown by the fact that in an *in vitro* experiment involving Pu EDTA/Tiron is indeed, superior to DTPA (Bruenger, F.W., Neilson, E.R. & Stevens, W., (University of Utah, C00-119-251, March 31, 1976).

It is probable that the use of metabolic inhibitors to reduce the rate of destruction of chelants would result in an enhancement of the ability to decorporate Pu and to treat toxic metal poisoning. I will be testing this approach in the coming months.

Another system under investigation for Pu decorporation is that of HEDTA/NTA. This combination from the chemical standpoint appears as effective as EDTA/Tiron for Th(IV) but neither HEDTA nor NTA are metabolically altered.

Finally, it is conceivable that the addition of DTPA to an MLC system involving EDTA or to HEDTA/NTA may very well prove superior, assuming that the pharmacokinetics of the two systems differ.

The results in Table 3 suggest that the most effective MLC - aside from metabolic considerations - would be one which utilizes the most effective primary chelant, assuming it came from an MLC. The apparent small effect of DTPA/Tiron relative to DTPA alone given in Table 3 may simply represent the fact that the DTPA, under the particular experimental conditions of early treatment, by itself ties up practically all of the available Pu. Therefore, no enhancement due to MLC formation can be manifested. This point is being checked by the use of smaller doses of chelants.

TABLE 3

Effect of Early, Multiple Administration
of MLC's on Monomeric Pu Decorporation^a

Treatment ^{b,c}	Pu Level % Inj. Dose ^d I.S.E.	
	Skeleton	Liver
Pu only (Early)	21.7 ± 1.9	27.9 ± 2.7
Pu only (Late)	39.6 ± 4.9	20.2 ± 2.6
EDTA	29.7 ± 2.6	20.2 ± 1.0
EDTA/Tiron	19.1 ± 1.8	12.7 ± 1.1
EDTA/PAS	32.6 ± 3.7	14.9 ± 1.8
EDTA/SHA	32.1 ± 2.5	15.0 ± 0.9
DTPA	10.1 ± 1.5	0.86 ± .23
DTPA/Tiron	9.7 ± 1.7	1.77 ± .26
DTPA/SHA	10.5 ± 1.8	0.65 ± .16

^aMonomeric Pu in 1% sodium citrate was injected intravenously (0.2 ml) into female, HA ICR mice (20 grams). Four to six mice per group were used.

^bTreatment was given i.p. twice daily for two days beginning two hours post Pu. The EDTA was given as the CaNa_2EDTA salt and that of DTPA as CaNa_3DTPA . The number of treatments and the doses are altered, depending on the decorporation effects of the primary chelant.

^cDoses were 0.25 mmols/kg for EDTA or DTPA and 1.0 mmol/kg for the secondary chelant.

^dThe mice were killed five days post Pu except for the "early" treated Pu which were killed two hours post Pu.

III. COMPLIANCE WITH CONTRACT REQUIREMENTS

We are carrying out systematic investigations on the relative effectiveness of selected single and mixed chelants for the decorporation of monomeric plutonium and in the treatment of poisoning in mice by toxic metals such as cadmium and mercury. The investigations have demonstrated that mixed ligand chelates can, indeed, be superior to the single chelants - one of the goals of this program. We have instituted a rapid screening method using automated potentiometric titration to detect mixed ligand chelate formation with sufficient stability to merit *in vivo* testing. The ultimate goal is to be able to treat poisoning and to decorporate insoluble forms of metals.

IV. EFFORT OF PRINCIPAL INVESTIGATOR

The Principal Investigator has, in fact, devoted about 75% of his time to the project compared to the 45% budgeted time.

V. ARTICLES PUBLISHED

Schubert, J. & Derr, S.K, Nature 275, 311-313 (1978); Schubert, J., Nature (in press).

Schubert, J., Riley, E.J., and Tyler, S.A., "Combined Effects in Toxicology--A Rapid Systematic Testing Procedure," J. Toxicol. Environ. Health 4, 763-776 (1978).