AN ABSTRACT OF THE THESIS OF

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Title: MERCURY AND CADMIUM BINDING PROTEINS IN MARINE MAMMAL TISSUES

Redacted for privacy

Abstract approved: Dr. D. R. Buhler

The subcellular fractions of liver and kidney from three bearded seals (Erignathus barbatus nauticus) and three walrus (Odobenus rosmarus divergens) were analyzed for cadmium and mercury content, and the results were compared with the results of subcellular fractionation of the same organs from two sea lions (Zalophus californianus californianus) obtained previously in our laboratory (Lee et al., 1976). When the heavy metal concentration in the cytosol was plotted against that in the whole cell, evidence for two phases of accumulation of heavy metals in the cytosol was obtained. At low cellular concentrations, proportionally more heavy metal was found in the cytosol than at higher concentrations. Under these latter conditions, cadmium had accumulated in the cytosol to a lesser degree, while mercury concentration remained constant.

The soluble fractions of kidney and liver from two bearded seals and three walrus were chromatographed on Sephadex G-75 and

cadmium and mercury distribution among proteins of different molecular weight was determined. Similar results for two sea lions (Lee et al., 1976) were compared with the ones for the arctic marine mammals. At low concentrations of heavy metals in the cytosol, the amount of metals in metallothionein was related much closer to that in the cytosol than at higher concentrations, where a very limited degree of linear correlation was observed. Two types of heavy metal binding sites with different affinities are a likely explanation for these biphasic distribution curves.

Mercury thus showed a distinct tendency to accumulate in the particulate fractions and the soluble proteins of high molecular weight, whereas cadmium was found much more in the soluble and metallothionein fractions.

Liver was recognized as the main organ to deposit mercury and kidney as a major site for cadmium accumulation in all three species. Nevertheless, the same general principle(s) appeared to govern heavy metal distribution in both organs.

A search in sea lion liver for a soluble protein of high molecular weight that binds mercury specifically failed to demonstrate such a protein, but showed that mercury binds nonspecifically to a great number of soluble proteins of around 150,000 daltons.

Use of a specific assay to measure metallothionein levels in livers and kidneys of three bearded seals, three walrus, and three

sea lions revealed a very low degree of linear correlation between metallothionein content and heavy metal concentration. Only cadmium levels in the kidney cytosol were correlated to some extent (R^2=0. 56) with the amounts of metallothionein found in the same organs. These results are inconsistent with the induction of metallothionein by high heavy metal concentrations in marine mammals.

The 10, 000 x g supernatant of sea lion liver homogenate containing an active mixed function oxidase system was found to catalyze the demethylation of added methylmercury in vitro at a rate of 22% in 16. 5 h.

Mercury and Cadmium Binding Proteins in Marine Mammal Tissues

by

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EXPLANATION OF ABBREVIATIONS AND SYMBOLS

BSA - bovine serum albumin

diMeHg - dimethylmercury, CH_3HgCH_3

DTE - dithioerythritol

EC - electron capture

g - gram(s)

GLC - gas-liquid chromatography

GSH - glutathione

G6PD - glucose-6-phosphate dehydrogenase

h - hour(s)

LD50 - lethal dose for 50% of the experimental animals

M - molar

mM - millimolar

μM - micromolar

MeHg - methylmercuric ion, CH_3Hg^+

min - minute(s)

mol - mole(s)

mmol - millimole(s)

μmol - micromole(s)

mV - millivolt(s)

MW - molecular weight

NAA - neutron activation analysis

NADP - nicotinamide adenine dinucleotide phosphate (oxidized)

NADPH - nicotinamide adenine dinucleotide phosphate (reduced)

nm - nanometer(s)

POPOP - p-bis-[2-(5-phenyloxazoyl)] benzene (secondary fluor)

PPO - 2, 5 diphenyloxazole (primary fluor)

R_f - distance compound moves/distance solvent front moves, or distance compound moves/distance tracking dye moves (gel electrophoresis)

sec - second(s)

TCA - trichloroacetic acid

TLC - thin layer chromatography

w/v - weight per volume

x g - times g, the gravitational force of the earth

MERCURY AND CADMIUM BINDING PROTEINS IN MARINE MAMMAL TISSUES

INTRODUCTION

Characteristics of Mercury as a Toxicant

Studying the action of a chemical towards living organisms involves many disciplines of science and requires knowledge in all these areas. Mercury is especially complicated in this respect because it forms many organic compounds, and because of its non-specific affinity to a great variety of biologically important molecules.

The biochemical aspect of the problem has been reviewed by Webb (1966) who gives the physical and chemical data of common mercury compounds and their effects on enzymes. The general pharmacology and pharmacodynamics of mercury and its compounds are covered by recent reviews by Clarkson (1972, 1973). The effect of methylmercury on humans, including symptoms of poisoning, recent case histories, and toxic levels, all viewed with an ecological background, is described in a short article by Kurland (1971). Jernelöv et al. (1975) have summarized the ecological aspects of mercury pollution with special emphasis on fresh water systems.

Very little of all this information will be repeated here to set the stage for the present studies. Figure 1 (Wallace et al., 1971) shows some of the forms and areas of occurrence of mercury in the

environment. Inorganic mercury is represented on the left side (phenylmercury and alkoxymercury compounds are of lesser concern for this study because of their limited occurrence, man-made origin, and eventual conversion to inorganic mercury). The right side shows the alkylmercury compounds, especially methylmercuric ion which is formed by bacterial methylation of Hg^{2+} in the aquatic environment. Methylmercury is the main mercury compound accumulated in the biomass. Because of its small size, lipophilic nature and high affinity for protein sulfhydryl groups, it facilitates virtually complete absorption, fast and uniform distribution over the body, and a long biological half-life (Table I). The half-life of excretion of methylmercury is 70 days in man and in some species of fish as high as 1000 days. As a result of its lipophilic character, methylmercury is known to cross the placenta and accumulate in the fetus whereas inorganic mercury does not. From its characteristics (Table I) it is obvious that methylmercury is the more dangerous compound in spite of its lower acute toxicity.

Aquatic organisms accumulate methylmercury and pass it on through the food chain. Therefore, man and other top carnivores can ingest elevated levels of this compound in their diet which then accumulates in their tissues until toxic symptoms occur as described by Kurland (1971).

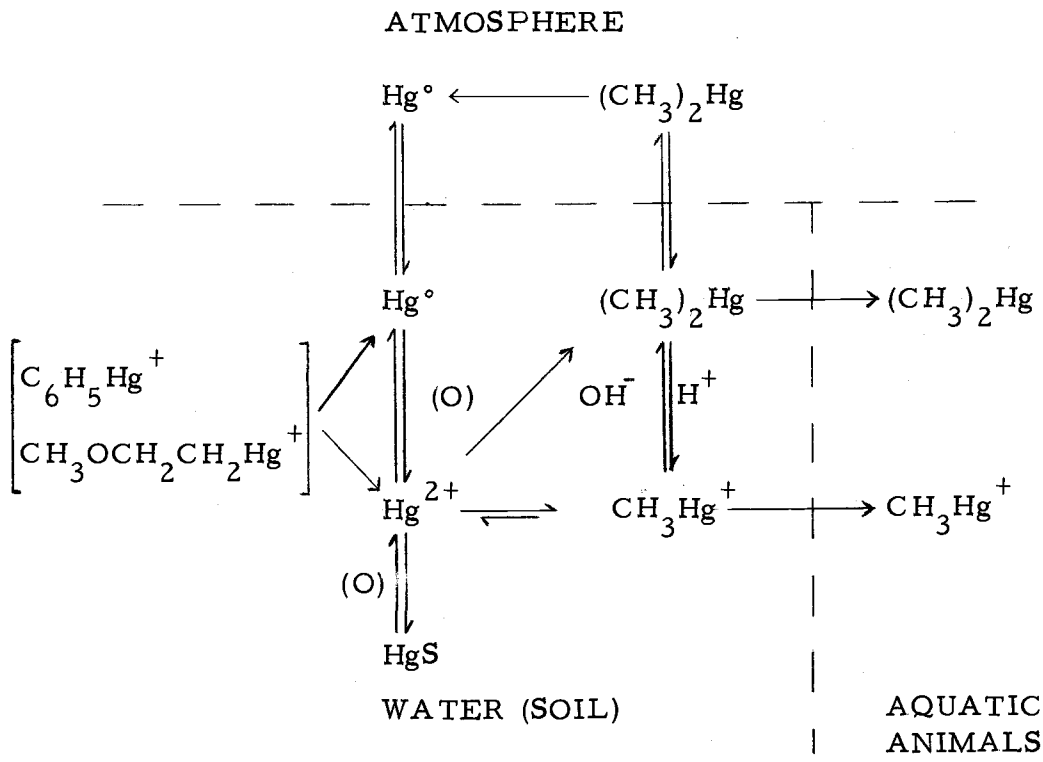

Figure 1. Some of the transformation pathways of mercury and its compounds in nature (Wallace et al., 1971).

TABLE I. COMPARISON OF THE BEHAVIOR OF METHYL AND INORGANIC MERCURY IN ANIMALS. The data are general trends observable throughout the literature.

	methyl-mercury	inorganic mercury
absorption	100%	15%
distribution	fast, even	uneven
accumulation	high	low
excretion	low	high
LD50 (i. p. mouse)	16 mg/kg	5 mg/kg

Cadmium as a Toxicant

No element can be seen alone in its effects upon living organisms, since nature consists of an intricate web of relationships. Cadmium is a heavy metal related to mercury through its similar electronic structure as apparent from the periodic system of elements. Like mercury, it is a very toxic element. A few examples of toxic effects of cadmium are discussed in the following articles: Carroll (1966) has correlated cadmium concentrations in the air of 28 American cities with the incidence of death from hypertension and arteriosclerotic heart disease; the same type of correlation has been made between cardiovascular death rates and cadmium levels in milk (Pinkerton and Murthy, 1969); chronic exposure to cadmium in humans is known to cause "itai-itai disease," characterized by severe pain in bones and joints, and kidney damage (Friberg et al., 1971); and necrosis of the gonads has been elicited in experimental animals by exposure to cadmium (Mason and Young, 1967; Parizek et al., 1968).

The toxicity of cadmium is counteracted by selenium (Mason and Young, 1967; Parizek et al., 1968). Selenium similarly protects against mercury toxicity (Parizek, 1971), a fact that stresses the relationship between the latter and cadmium. Though selenium may provide the answer to many of the questions in mercury toxicology, it will not be considered in this study for reasons of time and space.

Metallothionein--A Heavy Metal Binding Protein

Mercury and cadmium have similar electronic structures of their outer shells and, accordingly, similar affinities to a given ligand should be expected. Thiol sulfur, a functional group in many proteins, is such a ligand binding to both mercury and cadmium.

Metallothionein, discovered by Margoshes and Vallee (1957) in equine renal cortex, is a small protein (MW=6,000 to 10,000) containing roughly 30% cysteine. Pulido et al. (1966) have found 26 silver titrable SH-groups per molecule of human renal metallothionein. Normally, considerable quantities of zinc, copper, cadmium and mercury are bound to this protein. A biochemical role for metallothionein is not known, but its biosynthesis is inducible by pretreatment of laboratory animals with cadmium and less efficiently with mercury (Squibb and Cousins, 1974). For these reasons, this protein has been proposed to function as a vehicle of transport for and protective buffer against mercury and other toxic heavy metals (Friberg et al., 1971; Nordberg et al., 1971). Clarkson (1972) points out that "normal levels of metallothionein in rat kidney are equivalent in mercury binding capacity to a level of renal mercury associated with the onset of toxic effects." Therefore, it was argued that once the binding capacity of metallothionein has been saturated, toxic effects appear in the kidney.

The binding constants of different metals with metallothionein increase in the order: Zn^{2+}, Cd^{2+}, Ag^{+}, Hg^{2+} (Piotrowski et al., 1973a). This situation is quite analogous to the binding of these ions with inorganic sulfide. Protons compete for the SH-groups of metallothionein, displacing all the naturally binding copper, zinc and cadmium at low pH, whereas inorganic mercury binds strongly even under acidic conditions. Methylmercury does not bind to metallothionein in vivo (Chen et al., 1973; Buhler and Mate, 1975).

Protein-Binding of Mercury

In the cytosol of sea lion liver and kidney, mercury has been shown to bind to components of high and low MW. The low MW fraction has been identified as metallothionein (Lee et al., 1976). Metallothionein has also been found in grey seal, Pacific fur seal, and copper rock fish (Olafson and Thompson, 1974). No mercury binding proteins of high MW have yet been identified. But Chen et al. (1974) report the diversion of mercury in the cytosol of rat liver, testis, and kidney from metallothionein to a fraction of large MW due to pretreatment with selenium. Since selenium is found on the sea floor (Rosenfeld and Beath, 1964) and in marine fish (Lunde, 1972), the high mercury concentrations in the void volume fraction of a G-75 column of the cytosol from sea lion organs may constitute a phenomenon similar to that observed by Chen and coworkers which they termed a

"possible mechanism of protection."

Marine Mammals as Natural Test Organisms for Heavy Metal Contamination

The availability of tissue samples from individuals of three genera of pinnipeds [California sea lion (Zalophus californianus californianus), walrus (Odobenus rosmarus divergens), and bearded seal (Erignathus barbatus nauticus)] offered a chance to study the effects of unrestricted consumption of naturally high mercury and cadmium concentrations on warm blooded animals in high trophic levels of the food chain. The distributional zones of the three genera are spatially separated and undergo different characteristics of heavy metal input: sea lions living in the coastal waters of the North Pacific between Mexico and British Columbia (temperate), and walrus and bearded seals populating the Pacific side of the Arctic Ocean with particular abundance in littoral zones (arctic) (Scheffer, 1958; Walker, 1964). Differences in heavy metal contamination between the temperate and arctic species should be in part due to different concentrations of the contaminants in the waters of their habitat. Other factors involved are different feeding habits: the trophic levels in the marine food chain and the degree to which their food species concentrate heavy metals.

Sea lions are known to feed on fish and cephalopods in the water

column and at the bottom. The walrus is a bottom feeder living on mollusks and other benthic organisms. Occasional feeding on small whales and seals has been reported. The feeding habits of the bearded seal are controversial; older accounts (Scheffer, 1958; Walker, 1964) classify it as bottom feeder living on mollusks and crustaceans mostly, less frequently on fish. More recent opinions identify it as benthic to pelagic feeder (Federal Register, 1974; Mate, 1975).[1]

Demethylation of Methylmercury

Mean heavy metal contents found in each of the marine mammal species are given in Table II. There is more mercury by at least an order of magnitude in each organ of sea lion than in the same organ of the arctic species. Striking is the extremely high mercury concentration in sea lion liver, and the fact that the mercury is almost all in the inorganic form in this organ. Kidneys, as well, contain elevated mercury levels in the form of Hg^{2+}. The literature suggests that the principal form of mercury in fish, the food organism of sea lions, is methylmercury (Norén and Westöö, 1973; Buhler et al., 1973). This conveys the idea that sea lions may be able to demethylate methylmercury, which would result in facilitated excretion of this element meaning the possibility of reversal of the one-way

[1] Through personal communication with Dr. B. R. Mate, marine mammologist, School of Oceanography, Oregon State University.

TABLE II. HEAVY METAL CONTENT OF SOME MARINE MAMMALS

Tissue	Total mercury[a] (ppm)	Methylmercury (% of total)	Cadmium[a] (ppm)
3 California Sea Lions (No. 19, 20, 21)[b]			
Liver	95.7 ± 24.5	1.59 ± 0.64	1.61 ± 0.16
Kidney	5.43 ± 4.74	17.5 ± 9.0	7.22 ± 2.71
Muscle	0.84 ± 0.50	102.6 ± 10.7	0.085 ± 0.013
Heart	0.51 ± 0.34	61.9 ± 3.6	0.14 ± 0.05
Cerebellum	0.36 ± 0.15	40.5 ± 6.3	0.032 ± 0.002
Cerebrum	0.66 ± 0.48	28.5 ± 18.9	0.032 ± 0.006
Fat	0.19 ± 0.09	-	0.043 ± 0.015
5 Walrus (No. 1, 2, 3, 4, 5)[c]			
Liver	0.71 ± 0.18		7.70 ± 1.31
Kidney	0.16 ± 0.07		51.6 ± 35.5
Muscle	0.056 ± 0.044		0.45 ± 0.31
Heart	0.03 ± 0.009		0.152 ± 0.064
Cerebrum	0.037 ± 0.010		0.069 ± 0.021
3 Bearded Seals (No. 1, 2, 4)[c]			
Liver	2.7 ± 1.7		11.1 ± 10.2
Kidney	1.01 ± 0.64		43.4 ± 19.7
Muscle	0.045 ± 0.013		0.19 ± 0.11
Heart	0.04 ± 0.026		0.09 ± 0.03
Cerebrum	0.03 ± 0.006		0.046 ± 0.015

[a] Mean ppm (wet weight) ± S. D.

[b] From Buhler et al. (1975).

[c] From Buhler, unpublished results.

route of mercury into aquatic organisms (Figure 1).

Two quotes from a paper by Norseth (1972) may underline the importance of the demethylation reaction:

> Retention of small amounts of mercury over a prolonged period of time is the principal mechanism underlying the hazards of methyl mercury.

and:

> The release of methyl mercury may be important not only because of its relation to the excretion of mercury after methyl mercury exposure but also because its biotransformation may lead to changes in organ distribution with time. The biotransformation reaction may thus, by two different mechanisms, alter the target organ burden of mercury after methyl mercury exposure.

Research on other species has shown biodegradation of methylmercury to a limited extent *in vivo* in rats, mice, and guinea pigs (Clarkson, 1972; Lucier *et al.*, 1973; Garcia *et al.*, 1974a, 1974b). But the carbon-mercury bond in methylmercury is very stable, as *in vitro* demethylation catalyzed by tissue of experimental animals has never been reported, except in one case (Fang and Fallin, 1974) where minute percentages of Hg^{2+} were released from methylmercury by incubation with brain slices of rat. The rate of nonenzymatic demethylation (which has to be taken into account in evaluating an experiment) is 2.5% in 140 min in a 0.1M cysteine solution at pH 7 and room temperature (Norseth and Clarkson).[2] Since the body

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[2]The quoted article gives two incompatible values for nonenzymatic demethylation of MeHg: 2.5% in 140 min in a table of the data and 0.4% per day in the text. Personal communication with

temperature of the rat (37° C) lies 12 to 15° above room temperature, a three or more fold increase in the rate of nonenzymatic demethylation could be expected in vivo (van't Hoff's rule), which may account for the observed values.

Rationale and Goals

The reasons why sea lions were chosen for the studies described here, are summarized in the following:

1) They are on top of the marine food chain and mammals in these respects comparable to man.
2) Their principal food source is fish which contain methylmercury to 90% and above. Yet, in sea lion liver and kidney the main form of mercury is inorganic.
3) The mercury concentrations in sea lions are exceedingly high, however, the animals appear healthy.
4) Fossils of species similar to today's sea lions have been discovered in layers deposited in the Miocene (25 million years ago), and it is likely that these species were naturally exposed to mercury in the same way as today. Mercury has not only been introduced into the environment by man, but the natural levels exceed the man-made pollution by far.

Dr. T. Norseth (Yrkeshygienisk Institutt, Gydas vei 8, Boks 8149, Oslo 1, Norway) revealed that 2.5% in 140 min is the correct value.

> Human activities only result in local high concentrations. The selective pressure of relatively high mercury levels in their food may have been sufficient to develop a mercury metabolizing system in sea lions more so than in terrestrial animals.

Identifying this mercury metabolizing system and accompanying effects such as mercury and cadmium distribution in the main target organs liver and kidney, and mercury and cadmium binding to the components of the cytosol of these organs were the goals of the research efforts described in this thesis.

MATERIALS AND METHODS

Chemicals

Reagent grade chemicals were used throughout all experiments unless specified otherwise. Sources for chemicals were Mallinckrodt Chemical Works (St. Louis, Mo.), J. T. Baker Chemical Co. (Phillipsburg, N. J.), Sigma Chemical Co. (St. Louis, Mo.), Eastman Organics, Inc. (New York, N. Y.), Fisher Scientific Co. (Fairlawn, N. J.), and Matheson, Coleman, and Bell Manufacturing Chemists (Norwood, Ohio).

In all the laboratory work attempts were made to reduce possibilities of metal contamination. Glass redistilled water was used for all aqueous solutions and to thoroughly rinse all laboratory ware before use. All other solvents were obtained from the Department of Agricultural Chemistry, OSU as reagent or technical grade barrel stock and were distilled, if necessary, prior to use. Benzene for GLC was distilled over sodium. Toluene was distilled and repeatedly shaken with fresh batches of conc. H_2SO_4 until the aqueous phase remained colorless. Subsequently, it was washed with water and dried over Na_2SO_4.

Ammonium sulfate (ultrapure) was purchased from Schwarz/Mann (Orangeburg, N. Y.). Sephadex materials from Pharmacia Fine Chemicals Inc. (Piscataway, N. J.) were used for gel filtration.

DEAE-cellulose from Whatman Biochemicals Ltd. (Maidstone, Kent, England) was employed for all ion exchange chromatography, except for two unsuccessful trials with CM-Sephadex C50 and SP-Sephadex C50.

The matrix for specific ion mediated lipophilic chromatography (Rimerman and Hatfield, 1973) consisted of Sepharose 4B-200 and L-valine or DL-α-aminocaprylic acid from Sigma Chemical Co., or DL-norleucine from Mann Research Laboratories Inc. (New York, N. Y.).

Dr. P. Whanger, Dept. of Agricultural Chemistry, OSU, very generously furnished horse kidney metallothionein, that had been isolated to electrophoretic purity in his laboratory according to Kägi et al. (1974). Ghost shrimp, Callianassa californiensis, hemocyanin was kindly donated by Mr. Neal Eldred, Dept. of Biochemistry and Biophysics, OSU.

Cadmium (99.99% pure) as a standard for analysis was obtained from Fisher Scientific Co., dissolved in HCl/HNO_3 (1 g/7 ml 6 N HCl + 1.5 ml conc. HNO_3), and diluted with 1% HCl to make a standard solution of 500 μg cadmium/ml. The same results were obtained with a solution prepared from $CdCl_2 \cdot 2\,1/2\,H_2O$ (Mallinckrodt, AR) in 1% HNO_3.

Mercury standards were obtained from Mallinckrodt ($HgCl_2$, AR) and Alpha Ventron Co. (Beverly, Mass.) (CH_3HgCl, 95%). The

melting point of the methylmercury chloride was found to be 168.5 - 170.5° C (170° C indicated by producer).

New England Nuclear (Boston, Mass.) supplied the radioactive mercury compounds. $^{203}HgCl_2$ (radionuclidic purity 99+%) had a specific radioactivity of 1,017 mCi/mmol at the day of shipment. The compound was used without checking the manufacturer's specifications. The specific radioactivity of $^{14}CH_3HgCl$ was 4.17 mCi/mmol (radiochemical purity 98+%). The radiochemical purity of the ^{14}C-labeled methylmercury chloride was confirmed by TLC on alumina using chloroform:acetic acid:methanol (9:1:1) as solvent system.

Another method[3] to check the radiochemical purity of $^{14}CH_3HgCl$ including the results is depicted in Figure 2. With this method, one has to keep in mind that MeHg is demethylated in acidic cysteine solutions (Norseth and Clarkson, 1970) as confirmed by own experiments (see below). Therefore, during the extraction procedure some MeHg may be demethylated and the methyl carbon may end up in the aqueous phase. Most of the radioactivity in the final aqueous phase however, is probably due to incomplete extraction. This track has not been followed further.

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[3]Through personal communication with Dr. S. C. Fang, Dept. of Ag. Chem., OSU.

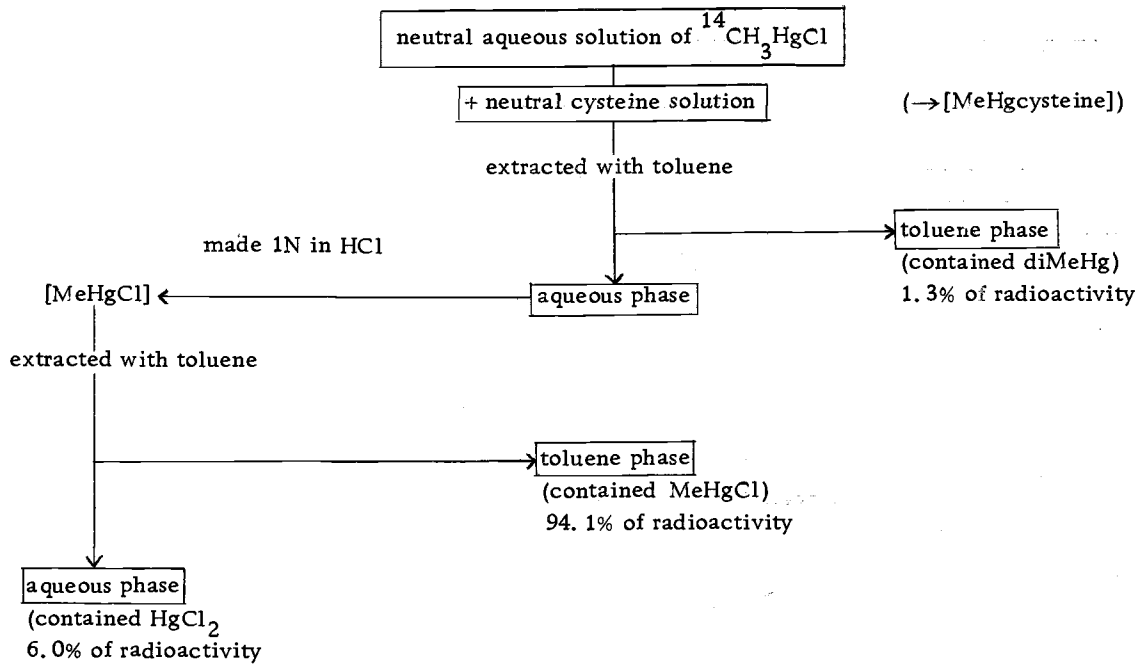

Figure 2. Test of radiochemical purity of $^{14}CH_3HgCl$

Animals

California sea lions, Zalophus californianus californianus, were obtained through Dr. B. R. Mate under permits issued to Drs. D. R. Buhler and B. R. Mate by the Oregon State Game Commission or the National Marine Fisheries Service. Site of sampling was mostly Simpson Reef, Cape Arago, at the Oregon coast.

Adult males (No. 19, 20, and 21) were humanely killed with a rifle on September 27, 1973, the needed organs excised in situ, chilled under ice within one to two hours, and used for experiments on the following day. The main portion of the tissues was kept

frozen at -20° C.

Two subadult animals, No. 22 female and No. 23 male both 1 1/2 years old, were captured on October 20, 1973 on Santa Cruz Island off the California coast. They were sacrificed November 3 and 5, 1973 respectively. Samples of all tissues were kept frozen at -20° C.

Livers and kidneys of bearded seals, Erignathus barbatus nauticus, and walrus, Odobenus rosmarus divergens, were collected by Dr. B. Mate in August 1973 on a research cruise in the Bering Sea aboard the research vessel "Alpha Helix" owned by Scripps Institute of Oceanography. The bearded seals are from the North Chuckchi Sea (our No. 1, 2, and 4 correspond to "Alpha Helix" sample No. 70, 72, and 74). The walrus were taken in the East Siberian Sea (our No. 1 through 5 correspond to "Alpha Helix" sample No. 64 through 68).

Random bred Wistar albino rats from a closed colony in the Department of Agricultural Chemistry, weighing between 200 and 400 g, were used as source for livers and kidneys in the initial and control experiments. To minimize variation due to sexual hormones in females (Palotta et al., 1962; Lucier et al., 1973), only males were used.

The rats were fasted overnight but received water ad libitum prior to being sacrificed by cervical dislocation and exsanguinated

by opening the carotid artery. Livers and kidneys were rapidly excised, washed free of blood with 1.15% ice-cold KCl solution and placed in beakers immersed in ice.

Homogenization of Tissues and Differential Centrifugation

Two methods of tissue preparation were applied with different success. For the subcellular fractionation in Part I as well as for the protein purification in Part II, frozen tissues were minced with a scalpel, weighed, placed in a mortar and completely covered with liquid nitrogen. They were ground to yield a fine powder while nitrogen was constantly refilled as it evaporated. The powder was weighed (usually about 1 g of tissue was lost in the process of grinding and transfer) and quantitatively rinsed into a polycarbonate centrifuge tube with 2 1/2 to 3 times its weight of prechilled buffer of 4° C. This procedure is discussed in Lee et al. (1976).

An alternative procedure used to prepare some of the incubation mixtures in Part III utilized a Potter-Elvehjem homogenizer with motor driven Teflon pestle according to Dewaide (1971). Homogenates (25-30% w/v) were prepared in ice-cold buffer in a cold-room at 4° C. Some of the tissue homogenates were used directly in the incubation mixtures in Part III and will be referred to as "whole tissue homogenates." At other times the homogenates were centrifuged at 10,000 x g for 30 min at temperatures between 0 and 4° C. The pellets were

discarded and only the supernatants (referred to as "10,000 x g supernatant") were incubated with substrate, following the assumption that the microsomal enzyme system was responsible for demethylating MeHg.

The subcellular fractionation of tissues into different cell organelles (Part I) was carried out according to previous practice in this laboratory (Lee et al., 1976). Thus direct comparison between the work on sea lions done by Dr. S. S. Lee and the present analyses on walrus and bearded seals is possible. The fractionation scheme is outlined in Figure 3. All centrifugation was done at a temperature between 0 and 4° C. For forces up to 48,000 x g, the superspeed RC2-B centrifuge with SS34 and GSA rotors from Ivan Sorvall Inc. (Norwalk, Conn.) was used. Above 48,000 x g, two centrifuges from Beckman Instruments Inc. (Fullerton, Calif.) were employed: Spinco L-2 ultracentrifuge with SW27 head and Spinco Model L centrifuge with Ti30 rotor. Resulting pellets were weighed, suspended in water, and aliquots treated further for determination of cadmium, mercury and protein.

In the experiments of Part II, after homogenization of the tissue, only one centrifugation at 48,000 x g for 40 to 60 min was carried out, the pellet discarded and the supernatant applied to a column or subjected to ammonium sulfate precipitation.

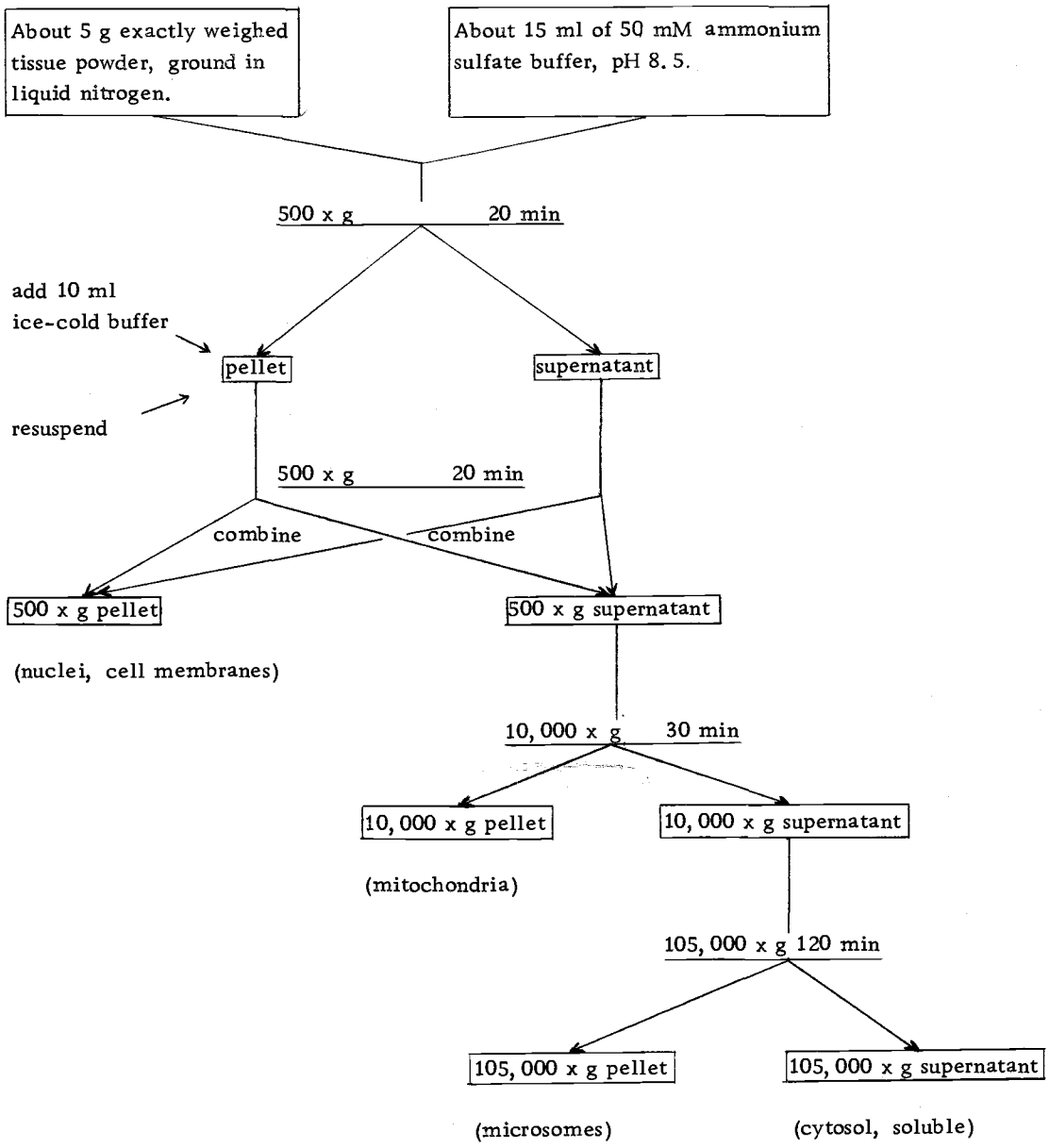

Figure 3. Schematic representation of fractionation of cell components.

Buffer-Solutions

A 50 mM ammonium sulfate buffer of pH 8. 5 was used in Part I to prevent dissociation of the cadmium-sulfur complex and to minimize interference with the determination of cadmium by atomic absorption.

The buffer for Part II, if not stated otherwise, was 20 mM phosphate at pH 7. 5 containing Na^+ and K^+ as counter ions. For the ammonium sulfate fractionation, 0. 2 M phosphate buffer, of pH 7. 5 was used for both the protein and the ammonium sulfate solutions to yield better buffering capacity and warrant a more stable pH during the precipitation.

Several buffers were employed in Part III for different experiments. An isotonic potassium phosphate buffer (26 mM, pH 7. 4) was the incubation medium for whole homogenate as well as 10, 000 x g supernatant of sea lion liver (stored frozen over prolonged periods of time) with mercury (400 ppm and 100 ppm) in the form of methylmercuric chloride.

Glucose-6-phosphate dehydrogenase used in the NADPH-generating system (see below) has been reported to be inhibited by phosphate (Negelein and Haas, 1935). According to these data, 26 mM phosphate should decrease the overall rate of NADP reduction catalyzed by the mentioned enzyme by 10-20%. This was the motivation to abandon phosphate as buffering ion in favor of Tris. Almost all the later

experiments utilized 50 mM Tris-HCl-buffer, pH 7.5 modified after an example from the literature (Dewaide, 1971). Only the last 2 of 12 experiments were conducted exactly according to Mazel (1971) in 0.25 phosphate buffer, pH 7.4 with Na^+ and K^+ as counter ions.

Digestion Procedure

For metal analysis, samples (whole tissue, subcellular fraction, or supernatant) were digested following a procedure worked out in Dr. D. R. Buhler's laboratory:

> Place up to 1 g sample in a flask under a condenser and add 5 ml of conc. HNO_3 through the condenser. Warm slightly to start the reaction. After the sample has dissolved, add 5 ml more of conc. HNO_3 and reflux without boiling for 3 h. Cool until just warm to touch and add 5 ml of 30% H_2O_2 in 1 ml increments. Boil the sample for 1 h (the brown NO_2 fumes should disappear), cool, rinse the condenser with redistilled water, and filter the sample through glass wool to remove the solidified fat. Record the volume and analyze aliquots for mercury and cadmium.

In samples treated this way, all the mercury was in the form of Hg^{2+}.

Protein Analysis

Protein in tissue samples and subcellular fractions (Part I) was precipitated with 10 ml of 0.2 N perchloric acid. After centrifugation, the 48,000 x g pellet was dissolved in 5 ml of 0.4 N NaOH and an 0.2 ml aliquot was analyzed according to the method of Lowry *et al.* (1951)

Lowry's method is biased in that it will underestimate a protein lacking aromatic residues. Metallothionein is known to contain only insignificant amounts of aromatic amino acids (Weser et al., 1973) or none at all (Kägi et al., 1974; Nordberg et al., 1972). Therefore, a turbidimetric method[4] was employed to estimate metallothionein and the proteins of high MW in Part II (Mejbaum-Katzenellenbogen, 1955). This method was standardized with BSA, so that only an approximation of the metallothionein concentration can be expected.

Piotrowski et al. (1973a) have published a specific method to estimate the metallothionein content in animal tissues. Radioactive inorganic mercury ($^{203}HgCl_2$) is added to unbuffered tissue homogenates with and without added metallothionein standard. All interfering proteins are precipitated with trichloroacetic acid. Metallothionein saturated with bound ^{203}Hg remains in solution and is then counted in a γ-counter. The difference in counts between the unspiked and the spiked sample (with a known amount of metallothionein added) is due to the metallothionein standard. With help of the weight and the radioactivity of the standard the amount of indigenous metallothionein is determined from the radioactivity of the unspiked sample.

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[4]Through personal communication with Dr. J. K. Piotrowski, Department of Toxicological Chemistry, Institute of Environmental Research and Bioanalysis, Medical School, ul. Naturowicza 120a; 90-145 Lodz, Poland.

For the experiment described in Part II, horse kidney metallothionein was used and the samples were scaled down by a factor of four. All γ-counting was done in the auto-gamma scintillation spectrometer, model 5230 by Packard Instrument Co. (Downers Grove, Ill.).

Cadmium Analysis

An atomic absorption spectrometer model 403 with graphite furnace and deuterium arc background corrector from Perkin Elmer Corp. (Norwalk, Conn.) was employed for cadmium analysis. A procedure to complex cadmium with ammonium pyrrolidine dithiocarbamate and extract it into methyl isobutyl ketone (Koirtyohann and Wen, 1973) was compared with the direct injection of column effluent or tissue digest. No interference with cadmium line absorption (2288 Å) of accompanying material was found and consequently, the samples were injected directly. The standard curve was linear from 5 to 100 pg cadmium. Samples of higher concentration were diluted accordingly.

Mercury Analysis

A short summary on mercury analysis by Claeys (1973) lists four methods for the analysis of mercury compounds: colorimetry, neutron activation, atomic absorption, and gas chromatography.

Radioisotope dilution analysis could possibly be added as a fifth method. But long incubation times of the radioactive mercury with the sample to obtain thorough equilibration have to be expected because of the high binding affinity of the tissue homogenates towards mercury. Different binding constants of mercury with different tissue components probably eliminate this approach altogether.

Colorimetry using dithizone is able to detect 0.02 ppm mercury in a 10 g sample (Smart, 1968) which is not sensitive enough by far to analyze column fractions, where only a small aliquot (0.5 ml to 1.0 ml) can be taken because the rest must be used for further steps. Feigl (1946) names copper-(I)-iodide as a sensitive reagent for Hg^{2+} (limit of identification: 30 ng Hg), but in tests to establish the usefulness of this chemical to dye the mercury associated with proteins in polyacrylamide gels, the detection limit of mercury was found to lie between 2 and 20 μg.

Neutron activation analysis seems very attractive at the first glance because of the high sensitivities achieved. De Soete et al. (1972) give 1 ppb as a concentration limit for detection and Mellinger and Smith (1973) claim 0.25 ppb in 1 g of biological material as limit for this method. On the other hand a critical interlaboratory study revealed NAA to be unsatisfactory for mercury in the ppb range (Heinonen and Suschny, 1972). But even with adequate sensitivity, NAA cannot be the method of choice because of several drawbacks:

the necessity of a nuclear reactor, the inability to discern different forms of mercury (unless an extraction procedure is employed in which case the convenience of not having to treat the sample is lost), the long irradiation times, and the long waiting periods before counting in order to allow for decay of interfering radionuclides (e. g. 15 h for ^{24}Na and 14 d for ^{32}P).

Cold vapor atomic absorption analysis combines the advantages of high sensitivity, specificity, speed of analysis, and low cost. The detection limit given in the literature is in the order of magnitude of 0. 5 ng mercury (Poluetkov et al., 1964; Magos and Clarkson, 1972; Least et al., 1974). The method is able to distinguish between inorganic mercury (Hg^{2+}) and methylmercury (CH_3Hg^+) in blood, urine, or column fractions of tissue homogenate supernatant without pretreatment of the sample (Magos and Clarkson, 1972).

The method of Magos and Clarkson with some modifications was used in this work. The analyzer system is represented in Figure 4. The light absorption of metallic mercury vapor was measured in a Coleman 50 mercury analyzer from Perkin Elmer Corp. (Norwalk, Conn.) and recorded by a Soltec recorder with integrator (Encino, Calif.). With a recorder sensitivity of 1 mV for a full scale deflection, 0. 5 ng mercury could very well be detected.

It was found that 50% $SnCl_2$ and 10% $CdCl_2$ (reductant used by Magos and Clarkson to measure total mercury) was not soluble in

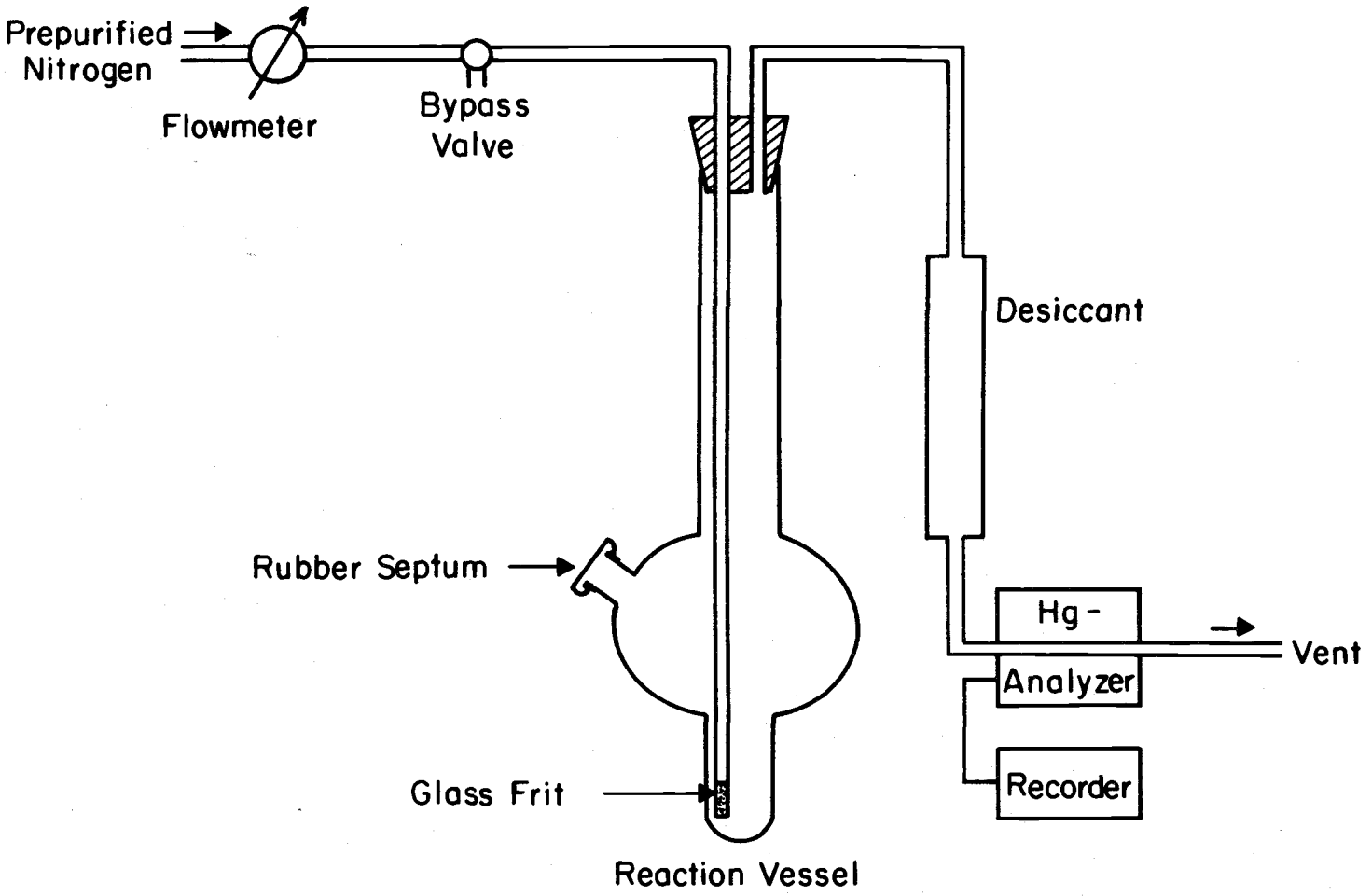


Figure 4. Schematic representation of the mercury analyzer system.

water. Rather than to use an aqueous suspension of $SnCl_2$ or lower its concentration, the reagent was made 4 N in HCl. This resulted in a clear solution.

Octanol (3 drops per reaction vessel) was added as an antifoam reagent. Sulfuric acid was omitted. For accurate determinations of total mercury, the sample and the reagents except 10 N NaOH were mixed, the apparatus assembled, 4 ml of 10 N NaOH were injected through the septum, and the mixture shaken. Nitrogen at a flow rate of about 850 ml/min was passed through the reaction vessel 30 sec after the injection. This initiated a peak in the recorder. Data were treated as described in Magos and Clarkson (1972).

For routine analysis of most of the column fractions and the tissue digests, a simplified and more rapid procedure was used. All the reagents (cysteine, reductant including cadmium, octanol, and base), but not the sample, were added into the reaction vessel and the gas was turned on. After all the mercury contaminating the reagents (blank) had been swept through the system and the recorder pen had returned to the baseline, three to four samples (not larger than 1 ml of volume) were injected followed by another standard. Between all the injections the pen was allowed to return to the baseline. The last standard showed that enough reducing potential and Cd^{2+} ion concentration had been available for the preceding samples.

There is a difference in rate of reaction of Hg^{2+} and CH_3Hg^+ with reductant. The Hg^{2+} is reduced instantly and results in a sharp peak. The mercury in MeHg is replaced by cadmium and then reduced. Substitution of cadmium for mercury is the rate limiting step and causes the peaks to flatten and broaden relative to the peaks brought about by the same amount of inorganic mercury. For this reason the area rather than the height of a peak was used as indicator of the total amount of mercury present in samples.

Analysis of spiked samples showed that with this modification, the recovery of inorganic mercury was complete and for MeHg it averaged 85%. In view of the great number of analyses (about 40 per column) and considering the fact that all the mercury in the digested samples and most of the mercury in the supernatants is inorganic (Hg^{2+}), the decision was made to use the simplified and accelerated procedure (20 analyses per h). Samples containing mainly MeHg were reanalyzed following the procedure modified after Magos and Clarkson (1972) as described first in this chapter.

Gas chromatography employing an electron capture detector is a very sensitive method for analysis of MeHg, with the detection limit reported for methylmercury chloride of 1 pg (Sumino, 1968). Hoover et al. (1973) found that mercuric chloride and MeHg displayed the same retention time under their conditions, the EC-detector being 500 times more sensitive towards MeHg. Therefore, GLC usually

is used for analysis of organomercurials, but one has to be aware of the possibility of interference by large quantities of Hg^{2+}. The advantage of GLC is high specificity for different organomercurials by virtue of different retention times. The disadvantage lies in the complicated extraction and clean up procedure, necessary especially if liver samples are to be analyzed.

In Part III of this study, GLC (Kamps and McMahon, 1972) was used to determine the decrease in MeHg due to demethylation as described below ("Chemical Analysis of Metabolites"). Since kidneys and especially livers of California sea lions contain very high concentrations of inorganic mercury (Table II and Buhler et al., 1975) but only about 1 ppm MeHg, it appeared much more promising to measure a decrease of MeHg content in the samples due to incubation, rather than an increase in the concentration of Hg^{2+} (the levels of which were very high from the beginning). This argument was supported by the fact that only low to moderate rates of demethylation were to be expected according to in vivo experiments conducted with other species (Norseth and Clarkson, 1970) and according to the relatively high levels of MeHg (0.5 ppm) found in sea lion muscle (Table II and also Buhler et al., 1975).

The method of Magos (1971) for measuring Hg^{2+} and MeHg one after the other in the same sample could not be adapted to this problem, and so MeHg was determined by gas chromatography.

Procedures of Protein Separation and Purification

All column chromatography was carried out at 4° C according to specifications of the manufacturers.

The columns for gel filtration had a bed volume of 700 ml, were 90 cm in length and of 2.5 cm inner diameter. The G-75 column was calibrated by Dr. S. S. Lee in our laboratory (Lee and Buhler, 1974).

Calibration of the G-200 column (Figure 13) was performed according to Andrews (1967). Marker substances and assay methods were:

1) blue dextran-2000 (800,000 daltons; Pharmacia, 1973) A_{600};
2) ghost shrimp hemocyanin (431,000 daltons; Roxby et al., 1974) A_{337};
3) beef liver catalase (240,000 daltons; Nicholls and Schonbaum, 1959) A_{406};
4) beef heart lactic dehydrogenase (140,000 daltons; Pesce et al., 1964) enzyme assay; and
5) sea lion hemoglobin (65,000 daltons) A_{540}.

Gel electrophoresis was carried out as described in Davis (1964). Later, modifications were made according to Brewer and Ashworth (1969). Column fractions were filled into dialysis bags. These bags were incubated overnight with crystalline sucrose. A sixfold decrease in volume was achieved and the samples now contained sucrose and were of sufficient density to be layered on top of the gel underneath the upper buffer. Sample volume for the

electrophoresis was 50 µl, but in case of mixtures of different fractions run in one gel, 15 µl of each fraction were applied.

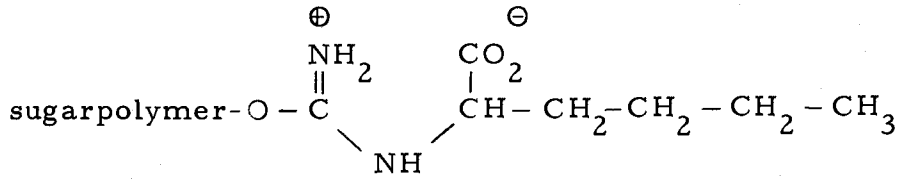
Gel slices were solubilized for mercury analysis with H_2O_2 (Grower and Bransome, 1970) and the excess H_2O_2 was decomposed with catalase. Protein in gels was estimated by comparing the absorption of a stained band in a densitometer with the absorption of a band created by a known amount of BSA in the same type of gel.

The procedure of Cuatrecasas (1970) as modified by Dr. R. A. Rimerman in our laboratory was employed to make the Sepharose derivatives for specific ion mediated lipophilic chromatography. An example (the batch of DL-norleucine-Sepahrose used for Figure 16) is described below:

> To prepare 200 ml norleucine-Sepharose, dissolve 40 g CNBr (well ventilated hood!) in 40-80 ml dioxane. Prepare an aqueous solution of 20 mmol (2.62 g) DL-norleucine in 200 ml 0.1 N $NaHCO_3$ brought to pH 10.0 with NaOH. Keep this solution just above freezing. Hold ready another 2 l of ice-cold 0.1 N $NaHCO_3$ solution of pH 10. Prepare a solution of high buffering capacity by making 500 ml of 5 M aqueous K_2CO_3 and adding as much (≈350 ml) 2 M aqueous $KHCO_3$ as needed for a final pH of 11.0. Take 200 ml Sepharose 4B-200 and wash out the preservative with water and then pH 11.0 buffer.
>
> Place the Sepharose into a beaker (reaction vessel) and add 200 ml pH 11.0 buffer. Stir and cool with ice. Measure the temperature and keep it between 24 and 25° C while adding the CNBr solution dropwise. Lowering the temperature too much decreases the rate of reaction, allowing it to increase too much will promote hydrolysis of the unstable CNBr-Sepharose intermediate. After adding the CNBr, let react for 10 more min, until the temperature drops below 23° C. Pour the content of the beaker into a Büchner funnel and wash with 2 l ice-cold 0.1 N $NaHCO_3$ solution of pH 10. Afterwards add the

norleucine solution into the funnel, stir, and pour the mixture into a different beaker. Stir overnight in the cold room. Wash the product thoroughly with water.

The product of the above reaction presumably has the following structure:



The charges on each site cancel, so that only the hydrophobic chain is effective. The longer this chain, the lower is the concentration of structure forming salt (von Hippel and Schleich, 1969) which is necessary to induce binding of a particular protein. More hydrophobic media will spread the more polar proteins of a mixture, whereas less hydrophobic media will separate the more lipophilic components of this mixture in a given decreasing salt gradient.

Incubation Procedure

Substrate (methylmercuric chloride) was incubated in concentrations between 8 and 400 ppm Hg relative to tissue. The higher concentrations were chosen because of the relatively high natural mercury content in sea lion livers (Table II), the lower levels because Norseth (1972) has shown a higher rate of biotransformation of MeHg in rat with low concentrations.

The MeHg was labeled with ^{14}C at the methyl group. Each test vial contained 46, 333 dpm, an activity sufficiently high as to allow the easy detection of a metabolite of only 1% of the total amount of substrate. In place of the substrate, two vials per experiment contained the same concentration of Hg^{2+} as nonradioactive $HgCl_2$ (standard).

Glutathione (GSH) is a tripeptide that supplies the cysteine residue in drug conjugation reactions, i. e. mercapturic acid formation (Boyland and Chasseaud, 1969) and is also a likely candidate to bind mercury. One objective was to determine whether or not GSH bound MeHg under the incubation conditions.

Because of the similarity in molecular size and shape between methionine and methylmercury cysteine, i. e.:

Methionine $CH_3-S-CH_2-CH_2-\underset{\underset{NH_2}{|}}{CH}-COOH$

Methylmercury cysteine $CH_3-Hg-S-CH_2-\underset{\underset{NH_2}{|}}{CH}-COOH$

it is conceivable that demethylation of MeHg follows the demethylation pathway of methionine. This idea especially suggests itself, since Landner (1971) has shown the opposite pathway (methylation of Hg^{2+}) to occur upon adding cysteine to a culture medium for Neurospora crassa containing inorganic mercury. MeHg-cysteine might be formed by enzymatic hydrolysis of MeHg-GSH.

DTE is an SH-stabilizing reagent (Cleland, 1964) that was always added to one set of samples to determine whether it was necessary to keep the sulfhydryl groups in the homogenate in the native, reduced state.

In vitro tests for some well studied microsomal demethylase activities (aminopyrine demethylase, ethylmorphine demethylase, p-nitroanisole demethylase) require NADPH as a cofactor. Consequently, in all but the first two experiments of this section, a NADPH-generating system (Mazel, 1971) was added to the incubation mixture. This system consisted of:

6.0 μg glucose-6-phosphate dehydrogenase (24.3 enzyme units),

0.3 μmol NADP,

11.5 μmol glucose-6-phosphate,

3.2 mmol $MgCl_2$,

and 32.0 μmol $MnCl_2$

in a total final volume varying between 2.2 and 2.7 ml in the individual experiments.

Mercuric ion in the rather high concentration of 1×10^{-4} M is known to completely inhibit G6PD (Glock and McLean, 1953). However, the enzyme would not encounter Hg^{2+} in this concentration in the incubation mixtures. No information concerning inhibition by MeHg could be found. Usually, the MeHg concentrations employed in the incubation mixture were not higher than 200 ppm relative to

tissue (20 μg Hg/ml of mixture). A short study revealed that 20 μg/ml of Hg as MeHg caused a 50% inhibition of G6PD (6 μg of protein in 2 ml). This much inhibition could be tolerated much easier than the high phosphate concentration of 0. 25 M suggested by Mazel (1971) to determine microsomal aminopyrine demethylase. According to Negelein and Haas (1935), 0. 25 M phosphate inhibits G6PD to 91%.

The mixture also included semicarbazide to trap any formaldehyde metabolite of the methyl group as the semicarbazone. After incubation, the mixture was cleared by centrifugation and Nash-reagent (acetyl acetone buffered to pH 6 with ammonium acetate) was added to quantitatively determine formaldehyde (Mazel, 1971).

The typical incubation mixture in a total volume of 2. 2 to 2. 7 ml contained:

1) Substrate (MeHg) or standard (Hg^{2+}) in concentrations discussed before.
2) GSH, 0. 55 μmol.
3) DTE, 10 μmol (every sample was run in duplicate, one vial with and one without DTE).
4) A NADPH-generating system.
5) Semicarbazide acetate, 36 μmol.
6) Whole tissue homogenate or 10, 000 x g supernatant corresponding to 100-250 mg of liver or kidney. Tissue blanks consisted of homogenate or supernatant which had been

denatured before they were introduced into the incubation mixture.

All the constituents of the incubation mixture had been made up in the same buffer before start of the experiment (see above, "Buffer Solutions"). These mixtures were incubated in dram vials or 25 ml Erlenmeyer flasks sealed air tight with rubber septums. Items 1) through 5) were preincubated at 37° C in a Dubnoff-type metabolic shaker for about 10 min. Following the preincubation period, the homogenate or supernatant was injected through the septum with a syringe. The reaction mixtures were shaken for aeration and usually incubated for 10 h, some for 30 h and longer.

The rates of in vivo demethylation of MeHg reported in the literature are rather slow (20% Hg^{2+} after 10 days in blood plasma of rats, 50% after 16 days in rat kidney--Norseth and Clarkson, 1970 and Lucier et al., 1973; and between 5 and 10% C-Hg bond cleavage in 24 h in various organs of the rat--Garcia et al., 1974). Consequently, attempts were made to convert measurable amounts of MeHg to Hg^{2+} by incubation with rat tissues for one to two weeks. The problem of bacterial putrefaction of the tissue homogenates was encountered in these experiments, and bacteria are reported to be able to demethylate MeHg (Tonomura and Kanzuki, 1969).

To overcome this problem, various antibacterial agents were added to theincubation mixture, but no demethylation was observed

under these conditions (0. 5% NaClO final concentration, neutralized with saturated boric acid--Manninger, 1940; 0. 02% NaN_3--Schwarting, 1946; and a mixture of 0. 4 μg penicillin and 5 μg streptomycin per ml--Baker, 1967). However, there was no way to tell whether these agents denatured or inhibited the demethylating enzyme system under investigation. For example, sodium azide is known to inhibit the catalase reaction (Keilin and Hartree, 1934) and the "synthesis of body compounds in favor of complete oxidation of nutrients" (Clifton, 1937).

Incubations were stopped by injecting 0. 5 ml of 25% $ZnSO_4 \cdot 7 H_2O$ and 0. 5 ml of saturated $Ba(OH)_2$.

To test for $^{14}CO_2$ as a presumptive metabolite of the methyl group from MeHg, the size of the reaction mixture was doubled and removable plastic wells (Neville and Feller, 1965) filled with 100 μl of a 1:1 mixture of redistilled β-phenethylamine and absolute ethanol (Woeller, 1961) were inserted into the air space above the incubation mixture and held by the rubber septum. In these experiments, the incubation was stopped by injecting 0. 4 ml of 6 N HCl immediately after 0. 9 ml of air had been drawn out of the sealed flask to facilitate the transfer of liberated $^{14}CO_2$ into the gas phase. After 30 min, the center wells were removed, the β-phenethylamine solution transferred into a counting vial, and the center wells rinsed twice with 0. 2 ml of fluor solution.

Chemical Analysis of Metabolites

A. Mercury

Decrease in MeHg concentration after incubation was measured by GLC as mentioned above under "Mercury Analysis." After acidifying the incubation mixture, MeHg was extracted into benzene, re-extracted into an aqueous cysteine solution, the solution acidified and extracted with benzene again (Westöö, 1968). At first toluene was used as organic phase for the extraction, until it was found that benzene was less contaminated with substances interfering with GLC and was thus easier to purify.

The final benzene extracts were injected (2 µl) into the gas chromatograph under the following conditions:

Instrument	:	Aerograph, HY-FI Model 600-D, Wilkens Instrument and Research, Inc., Walnut Creek, Calif. (now: Varian Aerograph)
Recorder	:	Sargent, Model SR
Column	:	Pyrex glass, length 5 3/4', outer diameter 1/8"
Liquid phase	:	Hi Eff 10 B
Solid support	:	Chromsorb W (Hi Pref) 80/100 mesh (Varian Aerograph)
Carrier gas	:	Prepurified nitrogen
Flow rate	:	25-30 ml/min
Detector	:	Tritium foil electron capture, Wilkens Instrument and Research, Inc.
Temperatures	:	Injection port : 210° C Column : 150° C Detector : 160° C

Under these conditions the retention time for methylmercuric chloride

was 54 sec and 200 pg Hg as MeHg gave a peak of 3. 7 cm at an attenuation of 8.

B. Metabolites of the Methyl Group

$^{14}CO_2$ was assayed as described above under "Incubation Procedure." Formaldehyde, GSH, and 3, 5-diacetyl-1, 4-dihydrolutidine, the product of the Hantzsch-reaction (condensation of acetyl acetone, formaldehyde, and ammonia--Mazel, 1971), were separated by paper chromatography (on Whatman No. 1 chromatography paper with the solvent system H_2O: H_2O-saturated phenol, 2:1). A standard of 3, 5-diacetyl-1, 4-dihydrolutidine was prepared on the paper by successively applying a solution of formaldehyde and Nash-reagent (see: "Incubation Procedure" above) on the starting line and drying at 60° C for 30 min. The R_f values in this system are 0. 90 for formaldehyde, 0. 77 for GSH and 0. 54 for the Hantzsch-product. Nash-reagent and ninhydrin were used to make the spots visible. The R_f values obtained with a mixture of these compounds compared well with the values obtained with each compound chromatographed by itself.

After denaturing the protein in the incubation mixtures, the precipitate was spun down (3, 000 rpm for 5 min in a clinical centrifuge, Model CL and rotor 221 from International Equipment Co., Needham, Mass.) and 10 µl of each supernatant applied on two

separate paper strips along with standard solutions of formaldehyde and glutathione. The chromatograms were developed as described above, sprayed, one strip with Nash-reagent, the other with ninhydrin, and scanned for radioactivity. A Packard Radiochromatogram Scanner System (Packard Instrument Co., Inc., Downers Grove, Ill.) Model 7200 with 4-π geometry was used. The settings of the instrument were:

Collimeter	:	2.5 mm
Time Constant	:	30 sec
Range	:	300
Scanning Speed	:	0.5 cm/min

No calibration relating peak area to dpm was made, because no radioactivity was recovered on the chromatograms.

In setting the conditions for counting the radioactivity of $^{14}CO_2$ and $^{14}CH_3HgCl$, Rapkin's review was used as a guide (Rapkin, 1973). All counting was done in disposable 1-dram vials filled to 4 ml total volume and inserted into standard counting vials in order to save scintillation fluid.[5] The fluor solution used for the β-phenethylamine samples and organic solutions (in toluene or benzene) was absolute toluene and absolute ethanol in a 2:1 ratio with 0.5% PPO and 0.01% POPOP. Aqueous samples were counted in Aquasol (New England Nuclear Co.). Samples (50 μl) were added to 3.95 ml of fluor in one vial and 50 μl of sample plus 50 μl of methyl-^{14}C-toluene of known

[5]According to advice of Dr. D. Reed, Dept. of Biochemistry and Biophysics, OSU.

radioactivity were added to 3. 9 ml of fluor in the second vial. Counting efficiencies were determined from the difference between the two counting rates after subtracting the background.

All counting was performed in a Packard Tri-Carb liquid scintillation spectrometer model 3375 (Packard Instrument Co., Inc., Downers Grove, Ill.) at optimal settings for ^{14}C.

RESULTS. PART I: DISTRIBUTION AND BINDING OF MERCURY AND CADMIUM IN TEMPERATE AND ARCTIC MARINE MAMMALS

Subcellular Distribution of Mercury and Cadmium

Both livers and kidneys from walrus No. 2, 4, and 5 and bearded seal No. 1, 2, and 4 were subjected to subcellular fractionation. Mercury, cadmium, and protein content was determined for each fraction. The same procedure was applied by Dr. S. S. Lee for sea lions No. 19 and 20 (Lee et al., 1976), and resulting data were used for comparison in this thesis. The results are shown in Tables III and IV.

All three species of marine mammals had higher mercury concentrations in liver than in kidney, but the cadmium concentrations in kidney exceeded those in the liver. This suggests that the liver is a target organ for mercury and the kidney a target organ for cadmium.

The most striking difference between walrus and bearded seal on one hand and sea lion on the other was that mercury levels were more than one order of magnitude higher in the livers of the temperate species, and generally higher in their kidneys as well (see also Table II). Interestingly, the arctic species showed higher cadmium levels than sea lions in both liver (5-20 ppm versus 1-2 ppm) and kidney (14-70 ppm versus 10 ppm).

In sea lion livers, mercury amounted to less than 5% in the cytosol (Table III). The bulk (above 60%) of the mercury was found

in the nuclear fraction. Nevertheless, the mercury concentration in sea lion liver cytosol was still higher than in the same fraction of the same organ in arctic marine mammals, but only about 10 times compared to 50 times in whole liver. Mercury in sea lion liver nuclei and cell debris was 40 times higher than in the arctic species with the exception of bearded seal No. 1. Liver mitochondria and microsomes of temperate animals contained 450 times and 150 times as much mercury respectively, as did the same fractions in arctic animals.

Subcellular distribution of cadmium in all specimens did not differ much from one another as did mercury distribution. In all of the analyzed animals, except sea lion No. 19, liver contained about 70% of the cadmium in the soluble fraction.

In bearded seal and sea lion kidney, 70% of the cadmium was also in the cytosol, while in the walrus about 55% was in that fraction (Table IV). Among different animals, increases in cadmium concentrations in the particulate fractions (nuclear, mitochondrial, microsomal) were more correlated with the increase in total tissue cadmium than with the increase in concentration in the cytosol (see APPENDIX I for analysis results for individual animals). This is expressed in the finding of lower percentages of cadmium in the soluble fractions in spite of higher absolute cadmium values. It seems therefore, that at a cadmium concentration in kidney between 25 and 50 ppm, the

TABLE III. SUBCELLULAR DISTRIBUTION OF MERCURY AND CADMIUM IN MARINE MAMMAL LIVER[a]

Species and Metal[b]	Fraction				Total
	Nuclear	Mitochondrial	Microsomal	Soluble	
Sea lion (2)[c]					
Hg (μg/g tissue)	38.1 ± 10.9	15.5 ± 4.9	4.70 ± 1.84	2.14 ± 0.09	60.4 ± 13.9
Hg (%)	62.6 ± 3.7	25.4 ± 2.3	8.4 ± 4.9	3.65 ± 1.06	100
Hg (μg/g protein)	748 ± 117	499 ± 275	7501 ± 4021	4.80 ± 0.84	115 ± 44
Cd (μg/g tissue)	0.45 ± 0.35	0.20 ± 0.18	0.08 ± 0.04	1.15 ± 0.80	1.86 ± 0.93
Cd (%)	21.9 ± 7.5	14.7 ± 17.0	5.35 ± 4.74	58.0 ± 14.1	100
Cd (μg/g protein)	7.75 ± 3.23	5.34 ± 3.93	120 ± 73	2.78 ± 2.36	3.62 ± 2.29
Bearded seal (3)					
Hg (μg/g tissue)	1.47 ± 1.28	0.07 ± 0.05	0.07 ± 0.06	0.73 ± 0.56	2.34 ± 1.78
Hg (%)	65.1 ± 11.6	3.8 ± 1.7	3.7 ± 1.9	27.4 ± 14.2	100
Hg (μg/g protein)	22.5 ± 21.5	21.7 ± 12.8	18.8 ± 14.8	7.4 ± 5.7	13.6 ± 10.9
Cd (μg/g tissue)	1.57 ± 1.22	0.14 ± 0.08	0.12 ± 0.02	6.00 ± 4.61	7.83 ± 5.92
Cd (%)	19.9 ± 0.5	2.0 ± 0.4	2.0 ± 0.9	76.2 ± 1.2	100
Cd (μg/g protein)	28.6 ± 22.0	30.6 ± 14.3	24.6 ± 16.0	40.9 ± 62.4	33.9 ± 37.9
Walrus (3)					
Hg (μg/g tissue)	0.42 ± 0.05	0.02 ± 0.01	0.06 ± 0.06	0.53 ± 0.04	1.03 ± 0.11
Hg (%)	41.4 ± 4.3	1.7 ± 0.5	5.0 ± 5.1	51.8 ± 3.8	100
Hg (μg/g protein)	13.0 ± 6.2	17.6 ± 19.4	17.1 ± 14.6	5.9 ± 1.1	7.9 ± 2.4
Cd (μg/g tissue)	2.35 ± 0.64	0.14 ± 0.12	0.20 ± 0.17	8.61 ± 2.46	11.31 ± 2.34
Cd (%)	21.3 ± 6.1	1.3 ± 1.5	2.0 ± 2.1	75.2 ± 9.1	100
Cd (μg/g protein)	74.6 ± 41.6	202 ± 293	63.2 ± 46.8	94.1 ± 18.1	84.9 ± 19.9

[a] Mean ±standard deviation; results for individual animals are listed in APPENDIX I.

[b] Number of animals in parentheses.

[c] From Lee et al. (1976).

TABLE IV. SUBCELLULAR DISTRIBUTION OF MERCURY AND CADMIUM IN MARINE MAMMAL KIDNEY[a]

Species and Metal[b]	Fraction				Total
	Nuclear	Mitochondrial	Microsomal	Soluble	
Sea lion (2)[c]					
Hg (μg/g tissue)	2.63 ± 2.99	1.07 ± 1.09	0.84 ± 1.10	1.28 ± 0.86	5.82 ± 6.05
Hg (%)	40.2 ± 9.7	19.0 ± 0.9	10.0 ± 8.6	30.9 ± 17.3	100
Hg (μg/g protein)	64.0 ± 74.6	39.3 ± 29.5	184 ± 237	4.5 ± 2.6	15.9 ± 15.8
Cd (μg/g tissue)	1.66 ± 0.37	1.65 ± 0.86	0.34 ± 0.26	8.65 ± 2.19	12.29 ± 3.69
Cd (%)	13.6 ± 1.0	13.0 ± 3.1	2.5 ± 1.4	70.9 ± 3.5	100
Cd (μg/g protein)	39.1 ± 11.7	68.9 ± 7.1	77.1 ± 50.8	31.0 ± 4.8	35.0 ± 7.1
Bearded seal (3)					
Hg (μg/g tissue	0.20 ± 0.11	0.03 ± 0.01	0.02 ± 0.02	0.45 ± 0.32	0.69 ± 0.45
Hg (%)	30.7 ± 3.3	5.5 ± 4.9	3.1 ± 1.1	60.7 ± 7.7	100 ±
Hg (μg/g protein)	2.04 ± 0.30	7.6 ± 10.8	14.1 ± 16.8	6.05 ± 3.73	3.55 ± 1.16
Cd (μg/g tissue)	8.46 ± 3.37	0.67 ± 0.51	0.86 ± 0.93	25.0 ± 10.2	34.85 ± 15.06
Cd (%)	24.5 ± 2.1	1.7 ± 0.6	2.0 ± 1.4	71.7 ± 2.6	100
Cd (μg/g protein)	22.5 ± 11.9	48.0 ± 27.8	64.7 ± 56.4	169 ± 236	79.0 ± 90.4
Walrus (3)					
Hg (μg/g tissue)	0.13 ± 0.02	0.02 ± 0.01	0.01 ± 0.00	0.16 ± 0.09	0.32 ± 0.09
Hg (%)	41.9 ± 8.6	6.0 ± 6.1	4.2 ± 2.0	32.2 ± 19.1	100
Hg (μg/g protein)	13.0 ± 6.2	17.6 ± 19.4	17.1 ± 14.6	5.9 ± 1.1	7.9 ± 2.4
Cd (μg/g tissue)	20.2 ± 9.18	2.58 ± 3.19	1.40 ± 0.89	30.1 ± 14.8	54.3 ± 25.5
Cd (%)	38.0 ± 7.0	4.1 ± 3.9	2.8 ± 1.4	55.1 ± 2.5	100
Cd (μg/g protein)	100 ± 92	116 ± 77	153 ± 149	232 ± 183	143 ± 63

[a] Mean ±standard deviation; results for individual animals are listed in APPENDIX I.

[b] Number of animals in parentheses.

[c] From Lee et al. (1976).

binding capacity of the cytosol is exceeded and the metal starts to accumulate in the cell organelles.

Summarizing one can say that all animals had high levels of heavy metals in liver and kidney, as was expected from their position on top of the marine food chain. Extraordinary high burdens in the case of mercury with sea lions had been channeled into the nuclear, mitochondrial and especially microsomal fractions of liver, and in the case of cadmium with the arctic species were bound to all kidney fractions equally. At low cadmium levels, this metal was overwhelmingly found in the cytosol.

Soluble Mercury and Cadmium Binding Proteins

The soluble fractions of liver and kidney from bearded seals No. 1 and 4 and walrus No. 2, 4, and 5 were chromatographed on Sephadex G-75 to determine the distribution of heavy metals with respect to MW. The fractionation of sea lion No. 19 liver supernatant on Sephadex G-200 (see Part II) was used for comparison with the arctic marine mammals. Also for the reason of comparison, the data from three G-75 gel filtration chromatograms (sea lion No. 19 kidney and sea lion No. 20 liver and kidney) were taken from Lee et al. (1976), recalculated to the present format, and are plotted in Figure 5 along with the same data from the bearded seals. The elution profiles from the supernatants of the walrus organs are shown in Figure 6.

Figure 5. Sephadex gel filtration of the soluble fraction from sea lion and bearded seal livers and kidneys. Total mercury (——); cadmium (- - -); and protein (A_{280}) (· · · ·). Sea lion No. 19 liver was chromatographed on G-200, all the others on G-75. The columns (dimensions as described in "Materials and Methods") were eluted by gravity with 50 mM ammonium sulfate buffer, pH 8.5.

In the G-75 columns, the first peak eluted is material above the exclusion limit (> 75,000 daltons), the second peak contains hemoglobin (65,000 daltons), the third peak (low in A_{280} and high in metal) contains metallothionein, and two peaks of low MW and low metal content follow. The G-200 elution profile shows two peaks of higher MW than hemoglobin. The distribution of mercury and cadmium between the void volume of G-75 and the metallothionein peak is indicated as percent of total recovery from the respective columns.

Data for sea lion No. 20 liver and sea lions No. 19 and 20 kidneys were taken from Lee et al. (1976) for comparison. Dr. S. S. Lee in our laboratory calibrated the G-75 column (Lee and Buhler, 1974), the calibration of the G-200 column is described in Results - Part II of this thesis.

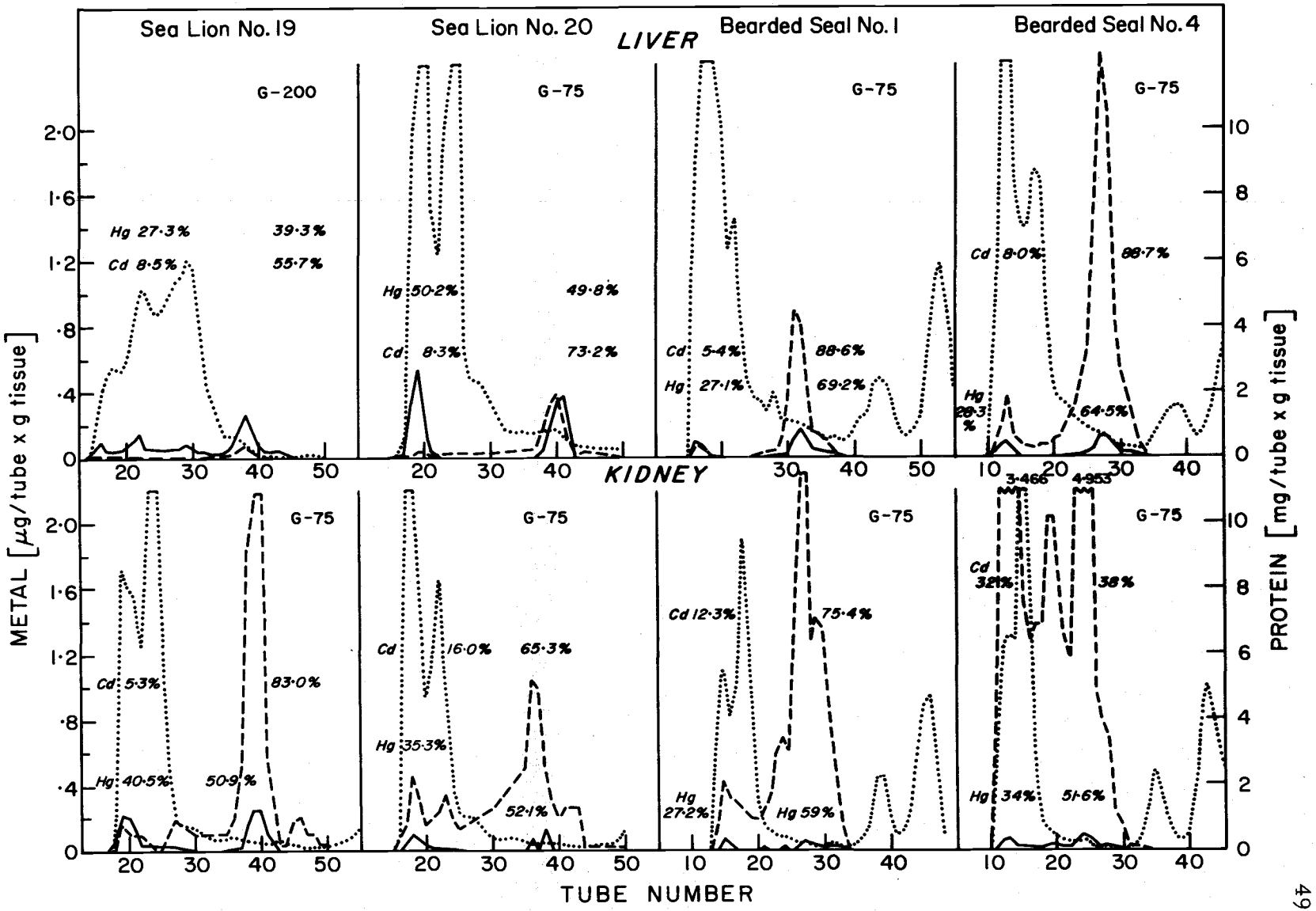
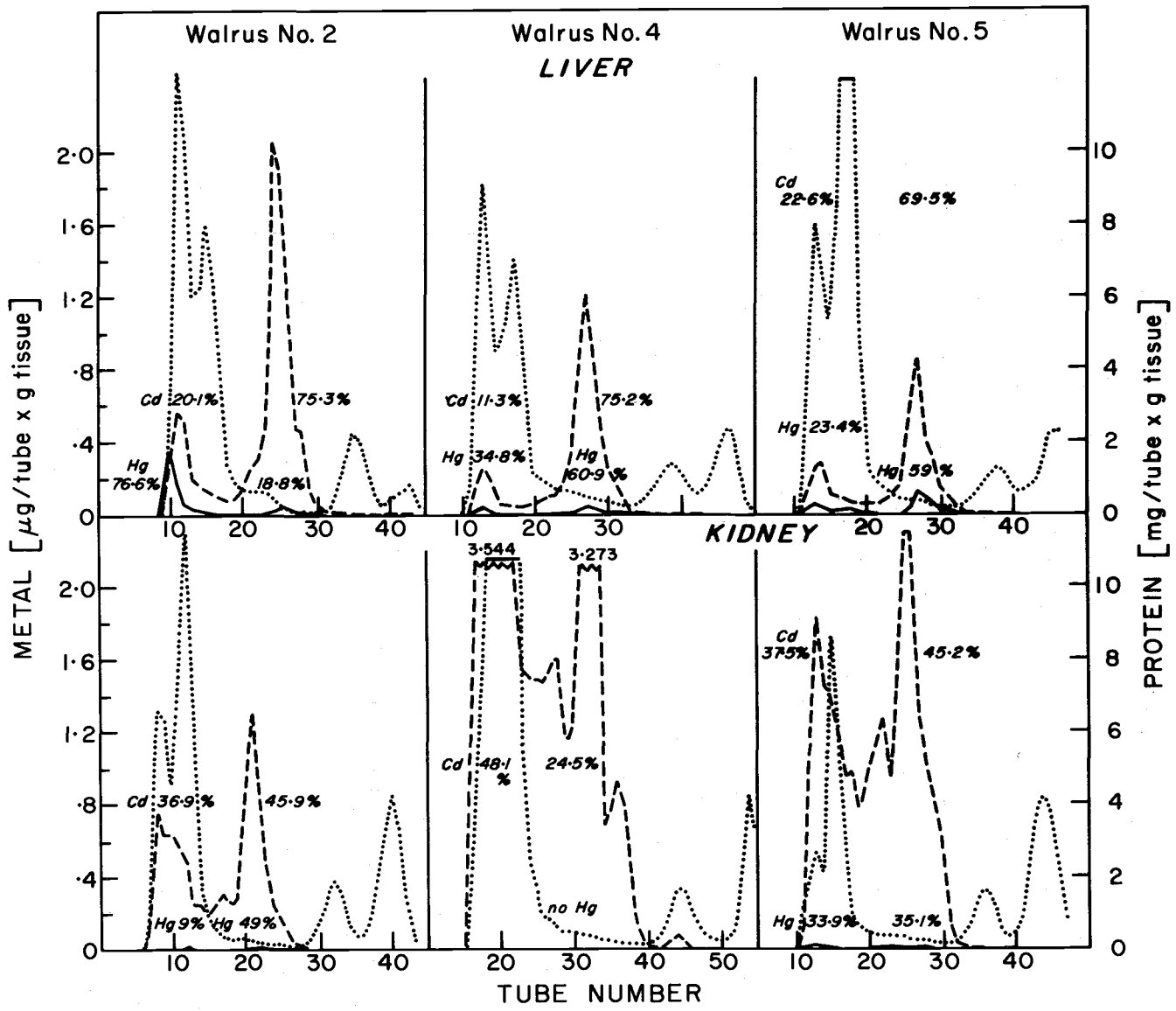

Sea Lion No. 19
Sea Lion No. 20
LIVER
Bearded Seal No. 1
Bearded Seal No. 4
G-200
G-75
Hg 27·3%
39·3%
Cd 8·5%
55·7%
Hg 50·2%
49·8%
Cd 8·3%
73·2%
Cd 5·4%
88·6%
Hg 27·1%
69·2%
Cd 8·0%
88·7%
Hg 28·3 %
64·5%
KIDNEY
Cd 5·3%
83·0%
Hg 40·5%
50·9%
Cd 16·0%
65·3%
Hg 35·3%
52·1%
Cd 12·3%
75·4%
Hg 27·2%
Hg 59%
3·466
4·953
Cd 32·1%
38%
Hg 34%
51·6%
METAL [μg/tube x g tissue]
PROTEIN [mg/tube x g tissue]
TUBE NUMBER

Figure 6. Sephadex G-75 gel filtration of the soluble fraction from walrus livers and kidneys. Total mercury (——); cadmium (---); and protein (A_{280}) (. . . .). The G-75 columns (dimensions as described in "Materials and Methods") were eluted by gravity with 50 mM ammonium sulfate buffer, pH 8.5.

The first peak eluted is material above the exclusion limit (> 75,000 daltons), the second peak contains hemoglobin (65,000 daltons), the third peak (low in A_{280} and high in metal) contains metallothionein, and two peaks of low MW and low metal content follow. The distribution of mercury and cadmium between the void volume and the metallothionein peaks is indicated as percent of total recovery from the respective column. The G-75 column was calibrated by Dr. S. S. Lee in our laboratory (Lee and Buhler, 1974).


Walrus No. 2
Walrus No. 4
Walrus No. 5
LIVER
KIDNEY
METAL [μg/tube x g tissue]
PROTEIN [mg/tube x g tissue]
TUBE NUMBER
Cd 20·1%
75·3%
Hg 76·6%
18·8%
Cd 11·3%
75·2%
Hg 34·8%
Hg 60·9%
Cd 22·6%
69·5%
Hg 23·4%
Hg 5·9%
Cd 36·9%
45·9%
Hg 9%
Hg 49%
3·544
3·273
Cd 48·1%
24·5%
no Hg
Cd 37·5%
45·2%
Hg 33·9%
35·1%

In all liver profiles, two heavy metal binding peaks dominated: the G-75 void volume and metallothionein. Metallothionein was not characterized in great detail for all the animals analyzed, but the very good reproducibility of its relative elution volume, the consistency of high values for metal analysis in conjunction with a low absorbancy at 280 nm, as well as absorbancies two- and more-fold higher at 250 than at 280 nm gave confidence that the observed protein was indeed metallothionein. It bound at least 70% of all the cadmium in liver supernatants with the exception of sea lion No. 19. This and the fact that the liver supernatants contained 70% and above of all the cadmium in the cell, means that about 50% of all the cadmium in liver was bound to metallothionein.

The other metal binding peak (G-75 void volume) usually was not as prominent as far as metals were concerned, but it was the highest A_{280} peak in most profiles indicating great amounts of protein. Only in the sea lion livers, this peak equalled metallothionein in heavy metal, i. e. mercury binding. As mentioned before, in sea lion livers mercury surpassed cadmium as prevalent heavy metal. However, 56 and 73%, respectively, of the soluble cadmium was found in the metallothionein fractions in sea lions No. 19 and 20.

The protein profiles of kidney supernatants showed the same number of peaks with the same MW distribution as the ones of liver. Generally, there was less mercury in kidney- than in liver-cytosol.

In some of the column fractions of walrus kidneys only traces of mercury were found and the scale in Figure 6 (equal for mercury and cadmium) does not permit this to be shown; only the inset figures inform about its relative distribution. In most cases, slightly more mercury appeared in the metallothionein fraction than in the void volume. Other fractions were negligible with respect to the amount of mercury bound.

Cadmium binding by the kidney supernatants appeared more divers than in liver, but a general principle could be recognized. In animals with only low cadmium concentrations (10 to 20 ppm) in their kidneys like the sea lions and bearded seal No. 1 (see APPENDIX I), from 65 to 83% of all the cadmium bound to the metallothionein fraction. In the specimens whose kidney was most highly contaminated with cadmium (walrus No. 4--72.8 ppm, walrus No. 5--70.1 ppm, and bearded seal No. 4--65.5 ppm), only 25, 45, and 39%, respectively, of the cadmium in the cytosol was bound to metallothionein. The other kidneys held intermediate positions.

The remainder of the cadmium in the cytosol was bound predominantly by the void volume fraction in kidneys low in cadmium. Supernatants from kidneys that were high in cadmium contained equal amounts of metal in all fractions of a MW higher than metallothionein.

Perhaps the principle of specific cadmium binding (to metallothionein) at low concentrations (around 10 ppm) and non-specific

cadmium binding at high concentrations (50 ppm and above) can be extended to livers as well. All the livers would fall in the low-cadmium category, and this would fit to the high percentage of cadmium binding to metallothionein in livers.

The results of the column chromatography in Figures 5 and 6 compare very well with the findings of Olafson and Thompson (1974). These authors show the elution profile of the 105,000 x g supernatant of grey seal liver homogenate passed through a Sephadex G-75 column. They obtained the same number of peaks with the same characteristics as shown throughout Figures 5 and 6. By appearance, their elution profile for grey seal liver fits into the low-cadmium class as observed in the present studies, however, no tissue cadmium concentrations were given in their paper.

Recoveries of heavy metals in the subcellular fractionation as well as the column chromatography varied appreciably. Many sources of error and variation due to the great number of treatments of the samples are obvious. In the case of incomplete or too high recoveries from the sum of the column fractions as compared with the analysis of whole supernatant, always the higher value which was more consistent with the whole tissue analysis was used and the deviating value corrected by a factor. Recoveries and corrections are shown in APPENDIX II.

Linear Correlation of Metal Concentration in the Metallothionein Fraction with Metal Concentration in Whole Tissue

It has been documented that metallothionein synthesis is inducible by oral or intraperitoneal pretreatment of laboratory animals with cadmium: in rabbits (Wiśniewska et al., 1970; Nordberg et al., 1972); rats (Shaikh and Lucis, 1970; Winge and Rajagopalan, 1972; Squibb and Cousins, 1974); and chickens (Weser et al., 1973b). As a result of these studies, it is now common practice to expose animals to elevated levels of cadmium if their tissues are to be used for the isolation of metallothionein. Piotrowski et al. (1973a) report a 10- to 40-fold elevated metallothionein content due to cadmium exposure in rat kidney and liver. In another publication, Piotrowski et al. (1973b) present evidence for an increased rate of metallothionein biosyntehsis in rat kidney due to long term exposure of the animals to mercuric chloride.

As a result of induction of metallothionein synthesis by heavy metals, one would expect to find higher amounts of heavy metals in the metallothionein fraction as the total tissue concentration of heavy metals increases. To test the validity of this hypothesis for marine mammals, total nanomoles of cadmium and mercury in the metallothionein fraction were plotted against the sum of whole tissue cadmium and mercury (Figure 7).

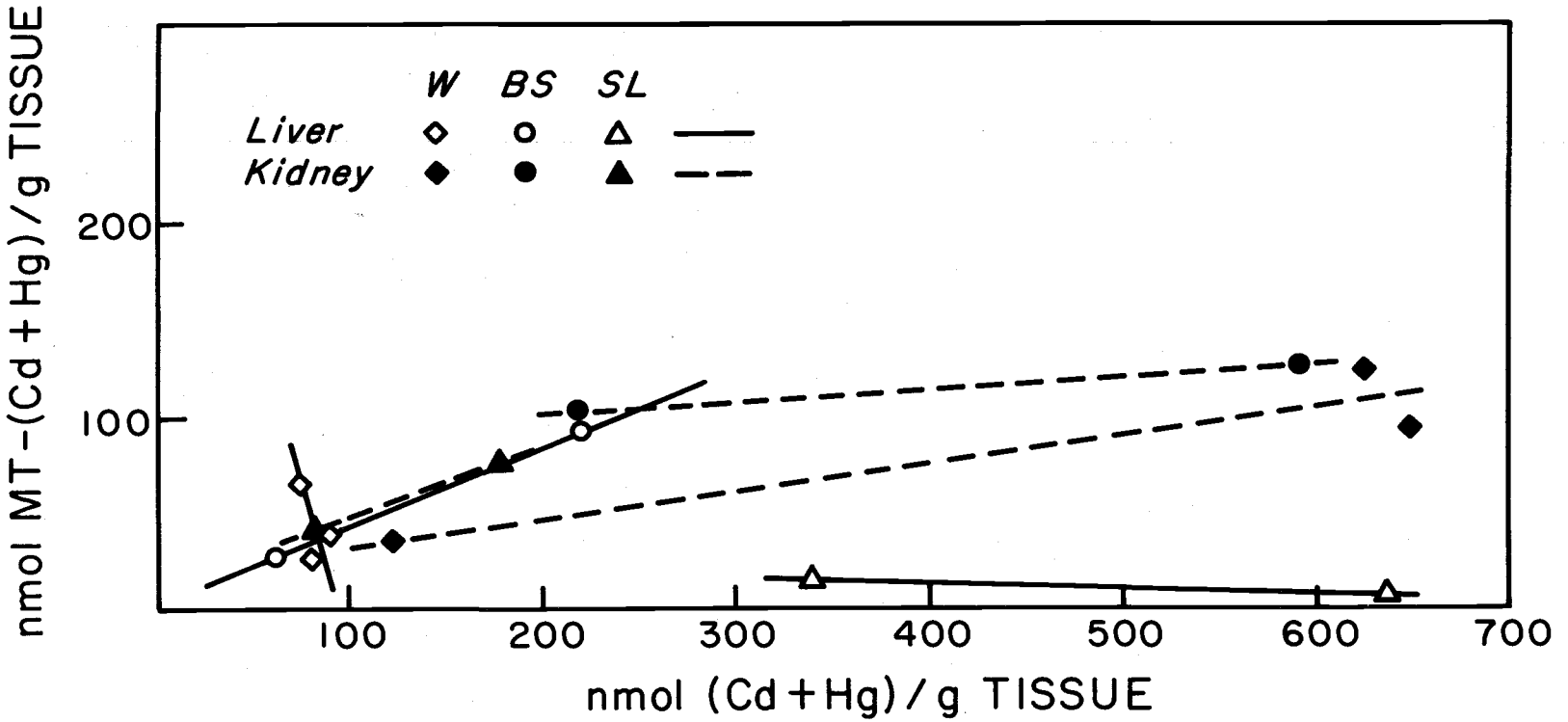


Figure 7. Correlation of heavy metal content in the metallothionein fraction with that in whole tissue. W = walrus; BS = bearded seal; SL = sea lion.

For most species and organs, a positive slope smaller than 1.0 was obtained. Sea lion kidney and bearded seal liver (60-220 nmol heavy metal/g tissue) exhibited slopes of about +0.4. The kidneys of the arctic marine mammals (120-650 nmol heavy metal/g tissue) showed slopes of around +0.1. These results suggest two phases of binding of heavy metals by metallothionein depending on heavy metal concentration, and are thus consistent with the hypothesis suggested previously in this thesis.

There was no correlation in sea lion livers (constant metal level in the metallothionein region in spite of varying tissue concentration) and in walrus livers (almost constant tissue concentration, but varying amounts of heavy metals bound to metallothionein).

It should be noted that in most of the analyzed samples from bearded seals and walrus, cadmium contributed by far the majority of the sum of heavy metals, and mercury added only insignificant amounts. In sea lion livers this relationship was reversed: the bulk of all heavy metals was mercury, and cadmium added only very little. As has been mentioned before, sea lion No. 19 was the only animal analyzed with less than 70% of all cadmium in the cytosol of liver cells associated with metallothionein (Figure 5). The same animal stood out with respect to its extremely high concentration of mercury in liver (125 ppm--Lee et al., 1976). Figure 5 shows that, only in this animal, mercury concentrations by far exceeded cadmium

concentratrations in all the fractions of the cytosol. Mercury with its higher affinity for SH groups may have blocked the cadmium binding sites and thus prevented absorption of greater amounts of this metal into liver cells. The same is true to a smaller extent for sea lion No. 20 liver with 65 ppm mercury (Lee et al., 1976). These facts may explain the outlier role of sea lion livers in Figure 7.

One has to be aware of the great danger of bias if only two data points, both subject to error, are used to draw a line. However, Lee et al. (1976) have shown a linear correlation between cadmium and mercury in the metallothionein fractions of G-75 gel chromatograms of California sea lion liver and kidney and the total cadmium and mercury concentration in these organs by analyzing as many as 5 different representatives of this species.

Possible Hypothesis Relating Cadmium Concentration in Metallothionein with Cadmium Concentration in Total Tissue

Liver levels of cadmium fluctuated between 1 and 20 ppm in all the animals, whereas cadmium levels were between 10 and 100 ppm in kidney. One possibility to explain the similar distribution of cadmium in liver and kidney tissues is that the total amount of cadmium in a tissue rather than the nature of the organ (liver or kidney) determines the distribution of metal.

Metallothionein has a great number of binding sites with high

affinity for cadmium. It is smaller than most biological macromolecules and occurs in the cytosol of the cell, therefore one might expect a relatively great mobility for this protein. These properties make it very likely that cadmium is bound by metallothionein when it first enters the cell. Later, cadmium is also bound by larger molecules in the cytosol due to exchange reactions. In the same way it reaches the cell organelles. If the binding capacity of metallothionein is exceeded, the distribution thus shifts towards a higher cadmium content in the less mobile and less accessible parts of the cell (large macromolecules, organelles).

Induction of metallothionein biosynthesis due to heavy metal exposure may or may not change the implications of this hypothesis. It will certainly raise the level of metal saturation of the metallothionein fraction, but will it prevent saturation?

Assumptions underlying these thoughts are negligible species differences in cadmium metabolism (all observable differences are due to cadmium intake) and the basic assumption of the heavy metal buffer theory, i. e. that metallothionein is not harmed or disturbed in any function by binding cadmium.

This hypothesis does not deny structural and functional differences at the subcellular level between liver and kidney, but it questions whether these differences are sufficient to cause the observed differences in cadmium distribution. It is through its function as a

blood filter that kidney may encounter more cadmium than does liver. The resulting question, whether cadmium distribution in liver would approach its distribution in kidney given similarly high concentrations, will have to be tested by specifically dosing liver with cadmium.

This idea of intrinsic similarity of cadmium distribution within liver and kidney cells was subjected to the quantitative test of regression analysis in the following section. This test was also applied to the mercury distribution data.

Regression Analysis

Figure 7 suggested that a biexponential curve of the type

$$MT = A_1 e^{B_1 T} - A_2 e^{B_2 T} \qquad (1)$$

could be fitted to the data [MT=nmol (Cd + Hg)/g tissue in the metallothionein fraction; T = nmol (Cd + Hg/g tissue; and A_1, B_1, A_2, and B_2 are parameters the best values for which are to be determined by regression analysis]. Consequently, a semilogarithmic plot was prepared (Figure 8a) and the data were subjected to regression analysis.[6] The first exponential

$$MT = A_1 e^{B_1 T} \qquad (2)$$

[6]The computer program "Exponential Curve Analysis" written by Donald A. Ramberg (Dept. of Ag. Chem., OSU) for the computer OS-3, was used for regression analysis of the data.

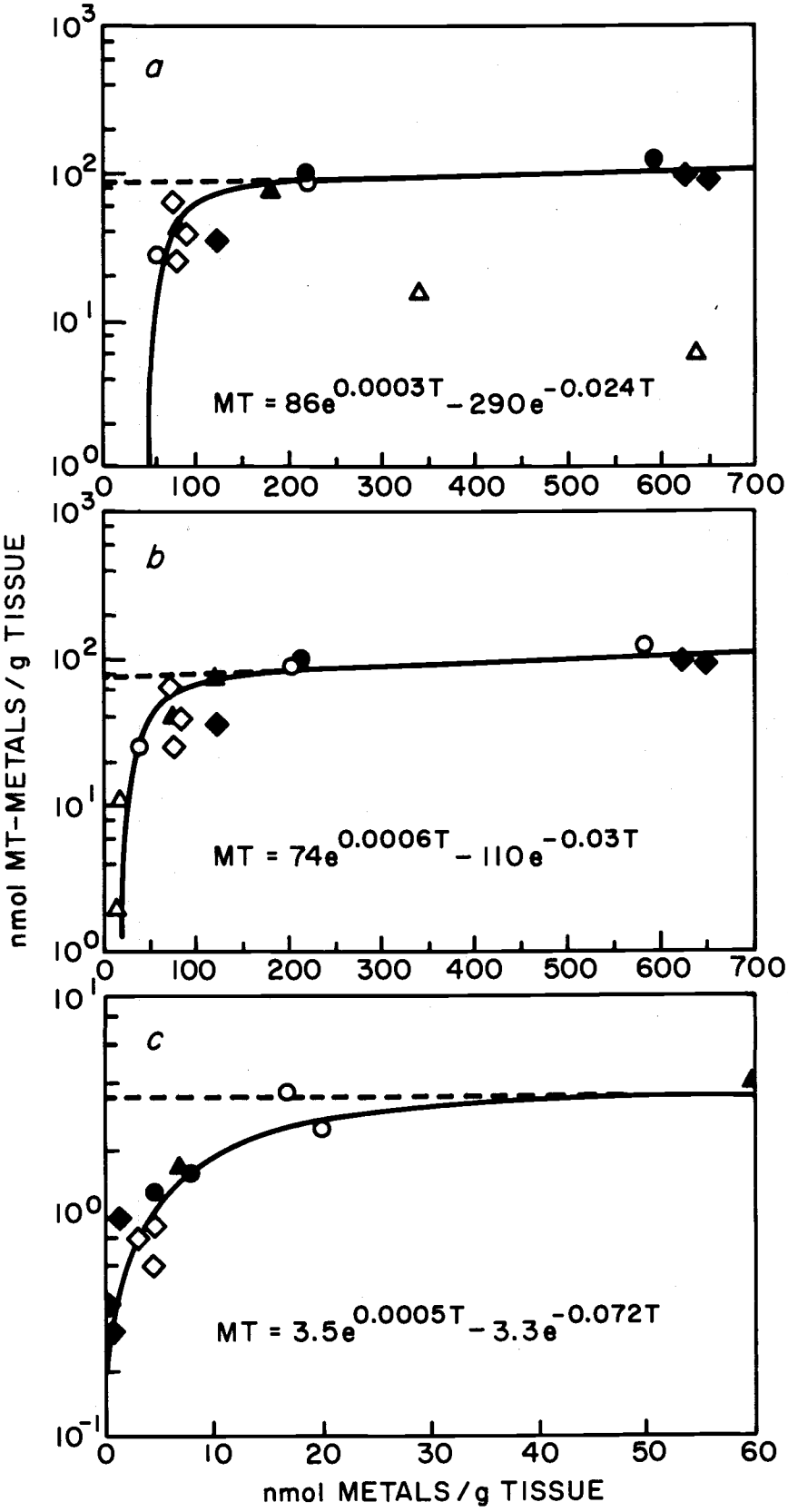
Figure 8. Approximation of the heavy metal distribution between metallothionein and the whole cell by biexponential curves.

a. Each point represents nmol (Cd + Hg) in one organ (liver or kidney) of one animal.

b. Each point represents nmol Cd in one organ of one animal.

c. Each point represents nmol Hg in one organ of one animal.

Liver and kidney, respectively: walrus (◇, ◆); bearded seal (O, ●); sea lion (△, ▲). The biexponential regression lines are shown as solid lines, the first exponentials (upper asymptotes) as dashed lines. Equations describing the regression lines are presented in the figure, with MT as nmol heavy metal/g tissue in the metallothionein fraction and T as nmol heavy metal/g tissue. The sea lion liver data are off scale in graph c, but were considered in constructing the regression line.


a
MT = 86e^0.0003T − 290e^−0.024T
b
MT = 74e^0.0006T − 110e^−0.03T
c
MT = 3.5e^0.0005T − 3.3e^−0.072T
nmol MT-METALS / g TISSUE
nmol METALS / g TISSUE

is plotted as a dashed line in Figure 8a. It represents one of the asymptotes of equation (1) and describes the amount of the metal binding to metallothionein at high metal concentrations in total tissue. At low metal concentrations in total tissue, the metal binding to metallothionein is described by the second exponential

$$MT = A_2 e^{B_2 T}. \tag{3}$$

The same sort of curve was fitted to the data describing the amounts of cadmium or mercury in the metallothionein fraction in relation to the total tissue concentration of these metals (Figure 8b and c). Dependence of the amounts of heavy metal bound to metallothionein (MT) on the heavy metal concentration in the soluble fraction (S) also was explored via such correlations (Figure 9a-c) as well as the heavy metal concentration in the soluble fraction relative to the total tissue level (T) of cadmium and (or) mercury (Figure 10a-c).

In all three cases (Figures 8-10), biphasic binding could be observed for cadmium as well as for mercury as well as for the stoichiometric sum of both. Figure 8 shows that with increasing heavy metal concentration in whole liver or kidney tissue (T), the heavy metal concentration in the metallothionein fraction (MT) increased rapidly, then slowed, and finally leveled off. The only drastic exception to this pattern was sea lion liver tissue containing exceedingly high amounts of mercury, which behaved atypically

(Figure 8a). This mercury was predominantly bound to the nuclear fraction (Table III), resulting in a lower degree of saturation of the metallothionein fraction (Figure 8c).

"Saturation" is the proper term only if the upper portion of the biexponential curve is horizontal. This is true if parameter B_1 in equation (1) equals zero, and it follows that parameter A_1 is identical to the saturation concentration of metallothionein with metals in this special case. Equation (1) then becomes

$$MT = A_1 - A_2 e^{B_2 T} \tag{4}$$

for $B_1 = 0$.

The numerical values for the parameters A_1, B_1, A_2, and B_2 for all the equations are given in the corresponding graphs (Figures 8-10) and in APPENDIX III, where also the 95% confidence intervals, R^2 and F values, as well as data points omitted for the regression analysis are listed. For the mathematical discussion of the biexponential curves, it is necessary to note that B_2 was negative in all cases. The 95% confidence intervals for the parameters B_1 indicate that a slope of zero for the upper asymptote or even a negative slope was included within experimental accuracy in all three graphs of Figure 8. Consequently, it is justified to talk of saturation or near saturation of the metallothionein fraction with cadmium at a tissue concentration of around 75 nanomoles per gram and with mercury at

a tissue concentration of about 3. 5 nanomoles per gram.

If the metal content of metallothionein (MT) was plotted against the amount of metals in the soluble fraction (S), regression analysis (Figure 9) yielded exponential functions of the same type as equation (1) fitting well to all data points not only for cadmium and mercury, but also for their stoichiometric sum. The upper asymptotes are more clearly ascending in all three plots of Figure 9 than in Figure 8. The possibility of horizontal lines as upper asymptotes in Figure 9a and b cannot be excluded with 95% probability. The second phase of mercury binding to metallothionein (Figure 9c), however, is dependent on the mercury concentration in the soluble fraction with 95% probability, but this may be due to limitations of the data. A glance at Figure 9c shows that the scale of the abscissa is expanded compared to Figure 9a and b. One might expect the curve in 9c to level off toward higher mercury concentrations in the soluble fraction.

Figure 10 shows the behavior of the metal concentration in the soluble fraction (S) as the concentration in total tissue (T) increases. The sea lion livers (Lee et al., 1976) were exceptional again, in that they contained extremely high amounts of mercury without corresponding high mercury concentrations in the cytosol. The cadmium concentration in the soluble fraction increased with cadmium in the whole cell (Figure 10b) in a biphasic manner without leveling off at high concentrations (no horizontal asymptote). The same effect was

Figure 9. Approximation of the heavy metal distribution between metallothionein and the soluble fraction by biexponential curves.

a. Each point represents nmol (Cd + Hg) in one organ of one animal.

b. Each point represents nmol Cd in one organ of one animal.

c. Each point represents nmol Hg in one organ of one animal.

Liver and kidney, respectively: walrus (◇, ◆); bearded seal (O, ●); sea lion (△, ▲). The biexponentials are shown as solid lines, the first exponentials (upper asymptotes) as dashed lines. Equations describing the regression lines are shown in the figure, with MT as nmol heavy metal/g tissue in the metallothionein fraction and S as nmol heavy metal/g tissue in the soluble fraction.

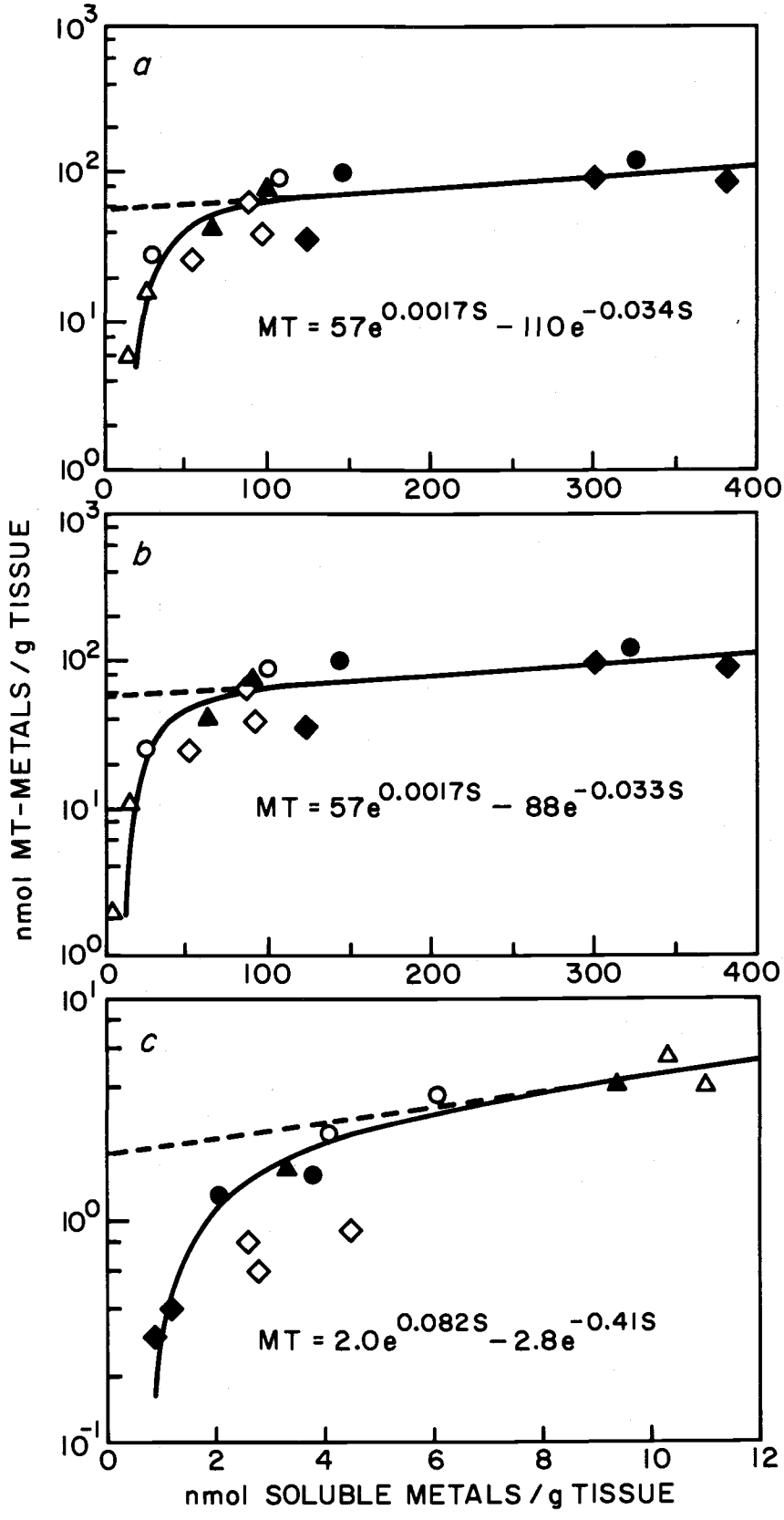

a
b
c
MT = 57e^0.0017S − 110e^−0.034S
MT = 57e^0.0017S − 88e^−0.033S
MT = 2.0e^0.082S − 2.8e^−0.41S
nmol MT-METALS / g TISSUE
nmol SOLUBLE METALS / g TISSUE

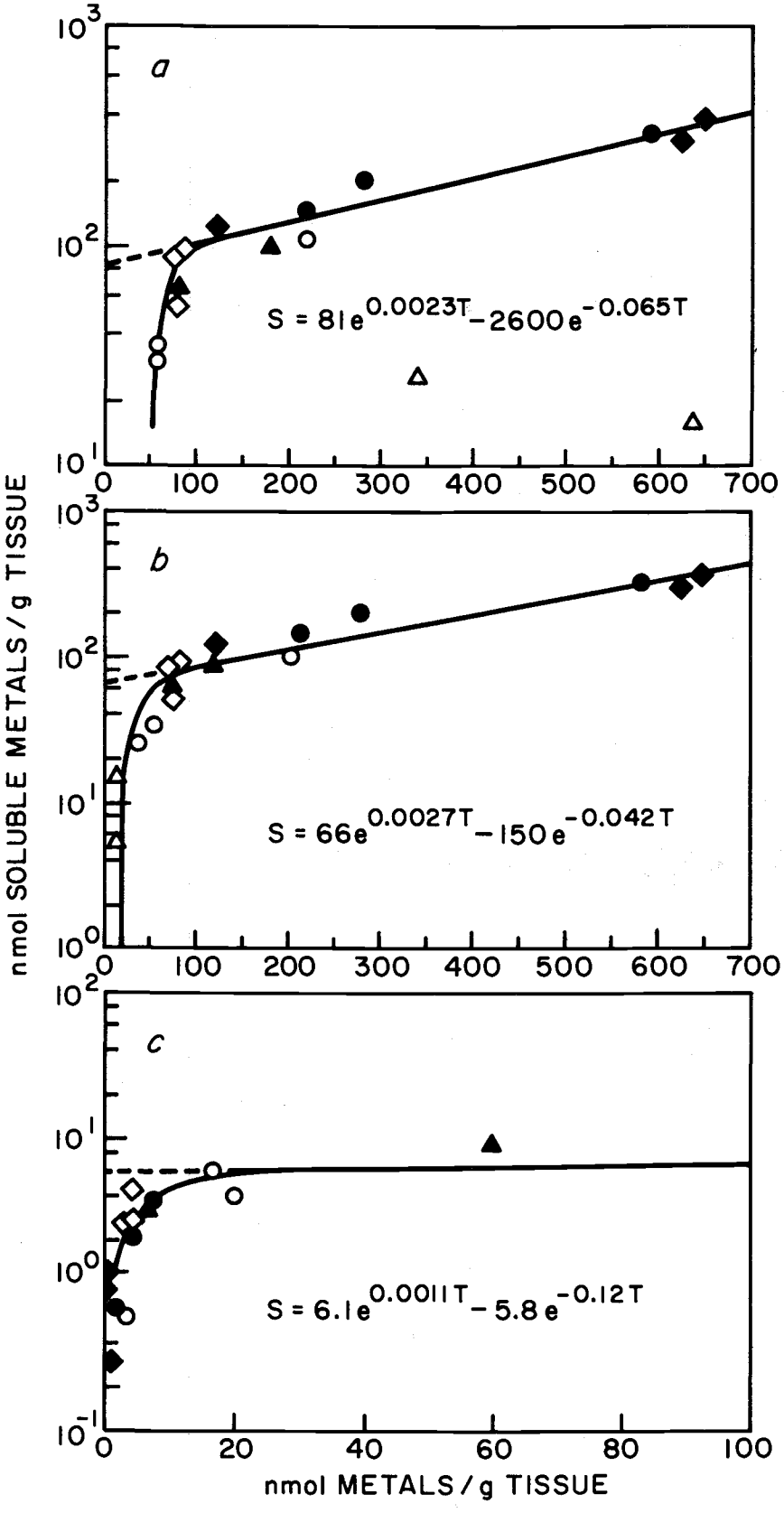
Figure 10. Approximation of the heavy metal distribution between the soluble fraction and the whole cell by biexponential curves.

a. Each point represents nmol (Cd + Hg) in one organ of one animal.

b. Each point represents nmol Cd in one organ of one animal.

c. Each point represents nmol Hg in one organ of one animal.

Liver and kidney, respectively: walrus (◇, ◆); bearded seal (O, ●); sea lion (△, ▲). The biexponentials are shown as solid lines, the first exponentials (upper asymptotes) as dashed lines. Equations describing the regression lines are shown in the figure with S as nmol heavy metal/g tissue in the soluble fraction and T as nmol heavy metal/g tissue.


a
b
c
$S = 81e^{0.0023T} - 2600e^{-0.065T}$
$S = 66e^{0.0027T} - 150e^{-0.042T}$
$S = 6.1e^{0.0011T} - 5.8e^{-0.12T}$
nmol SOLUBLE METALS / g TISSUE
nmol METALS / g TISSUE

shown by the sum of cadmium and mercury (Figure 10a). This is not surprising, since the order of magnitude of the mercury concentration in all but sea lion livers was not sufficient to change the trend of the cadmium concentrations. In the case of mercury distribution, the soluble fraction appears to be saturated at 6 nanomoles per gram tissue as indicated by the horizontal asymptote in Figure 10c. In this figure, two points (sea lion livers) are beyond the scale of the graph but were considered for the regression analysis. They fit well to the horizontal line.

In all these plots (Figure 8 through 10) we encounter a common phenomenon: a system (the cell or the cell cytosol) accumulates heavy metals by first filling one of its compartments (the cytosol or the metallothionein fraction) at a faster rate than the other compartments. With the accumulation of a higher heavy metal concentration by the system, the rate of accumulation of heavy metals in the special compartment decreases drastically, and in some cases approaches zero.

With metallothionein as an example for the special compartment and the whole cell or the supernatant as the corresponding system (as illustrated in Figures 8 and 9), three different explanations can be formulated for the initial rapid rate of increase: heavy metals bind to metallothionein as fast as they enter the cell, or more metallothionein is synthesized as more heavy metals reach the cell, or an overlapping of both processes takes place. A surprising result is

the near horizontal slope of the upper asymptote indicating either saturation of the preformed metallothionein with metals and no induction of its biosynthesis, or decrease of metallothionein biosynthesis due to high concentrations of heavy metals; or binding of heavy metals to other parts of the cell with greater affinity or accessibility than metallothionein. It is noteworthy that above 100 nanomoles heavy metals per gram tissue, most of the cadmium binds to the cell organelles, and the cadmium concentration in the cytosol increases only slightly (Figure 10b). This slight increase in the cytosol is not reflected by a corresponding increase in metallothionein-bound metal (Figure 9b).

If the cytosol is regarded as the special compartment and the whole cell as the whole system as shown in Figure 10, the two different phases of binding can be interpreted as due to two types of binding sites with different affinities for heavy metals.

An entirely different approach can be chosen by plotting the metal present in the whole system against that in the special compartment as shown in Figure 11. These semilogarithmic plots yielded straight lines in all three cases for cadmium as well as for mercury. Plotting mercury and cadmium on the same scale as shown in Figure 11, very clearly revealed the tendency with increasing mercury concentrations in the cell for mercury to accumulate in the particulate or higher MW fractions. Cadmium accumulated in the soluble and

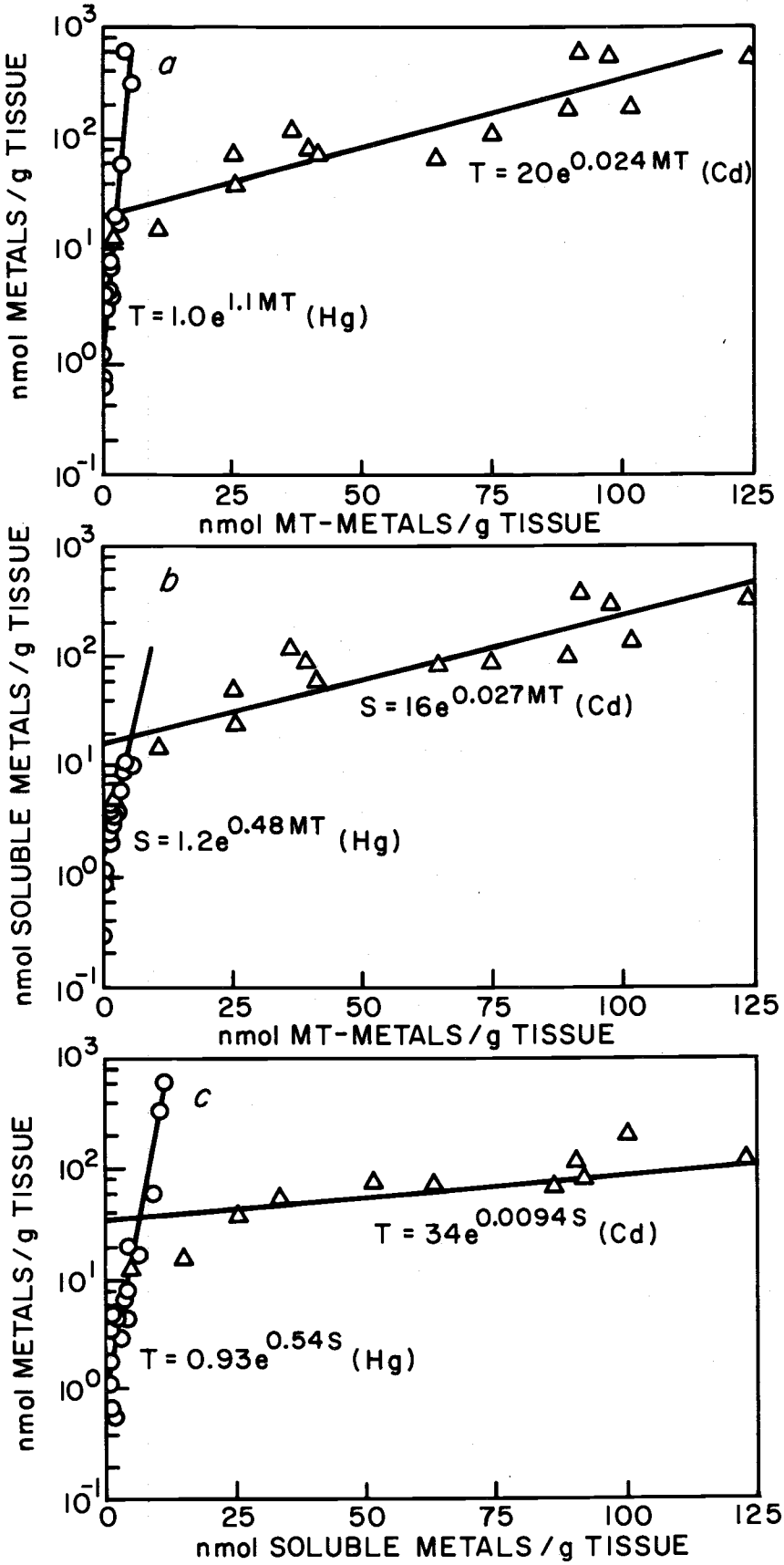
Figure 11. Approximation of the heavy metal distribution by exponential curves.

a. Metals in tissue plotted against metals in metallothionein.

b. Metals in the soluble fraction plotted against metals in metallothionein.

c. Metals in tissue plotted against metals in the soluble fractions.

Mercury (O) and cadmium (△). Each point represents one organ (liver or kidney) of one animal. Equations describing the regression lines are shown in the respective figures, with T as nmol heavy metal/g tissue, MT as nmol heavy metal/g tissue in the metallothionein fraction, and S as nmol heavy metal/g tissue in the soluble fraction. Points are not identified according to organ and species because of heavy clustering. In Figure c, the points for cadmium in the three bearded seal kidneys and walrus No. 4 and 5 kidneys are beyond the scale but were considered in constructing the regression line.


a
T = 20e^0.024MT (Cd)
T = 1.0e^1.1MT (Hg)
nmol METALS / g TISSUE
nmol MT-METALS / g TISSUE
b
S = 16e^0.027MT (Cd)
S = 1.2e^0.48MT (Hg)
nmol SOLUBLE METALS / g TISSUE
nmol MT-METALS / g TISSUE
c
T = 34e^0.0094S (Cd)
T = 0.93e^0.54S (Hg)
nmol METALS / g TISSUE
nmol SOLUBLE METALS / g TISSUE
0
25
50
75
100
125

metallothionein fractions (special compartments) to a much greater extent than mercury as the shallower slopes of the cadmium lines in Figure 11 indicate. These results suggest a relationship of the type

$$y = Ae^{Bx} \tag{5}$$

where y designates the system, x the special compartment, and A and B are parameters. The example of equation (5) that is directly comparable with equation (4) is

$$T = Ae^{BMT} \tag{6}$$

where A and B are parameters different from A_1, A_2, and B_2 in equation (4).

Equation (5) fits the data quite well as can be seen in Figure 11 and in APPENDIX III where the statistical parameters are listed. But mathematically it is impossible to derive equation (5) from equation (4). Solving equation (4) for the independent variable (T) yields:

$$T = \frac{1}{B_2} \log \left(\frac{A_2}{A_1 - MT}\right). \tag{7}$$

Equation (7) has an asymptote where A_1 equals MT. Equation (5) does not have an asymptote which shows the incompatibility of the two mathematical expressions. Both expressions, however, fit the data. The conclusion is that the data are too limited in range and

scattered too much to allow a decision in favor of either equation.

Two ways to decide between equations (4) or (7) on one hand and (5) on the other are possible: either laboratory experiments dosing marine mammals with cadmium and mercury above their natural levels to generate more extreme values; or more analyses of the type described here (Tables III and IV and Figures 5 and 6) to gain more confidence by averaging out individual variations and errors of analysis. The second approach, of course, is much more feasible.

RESULTS. PART II: HEAVY METAL BINDING PROTEINS OF HIGH AND LOW MOLECULAR WEIGHT

Gel Filtration

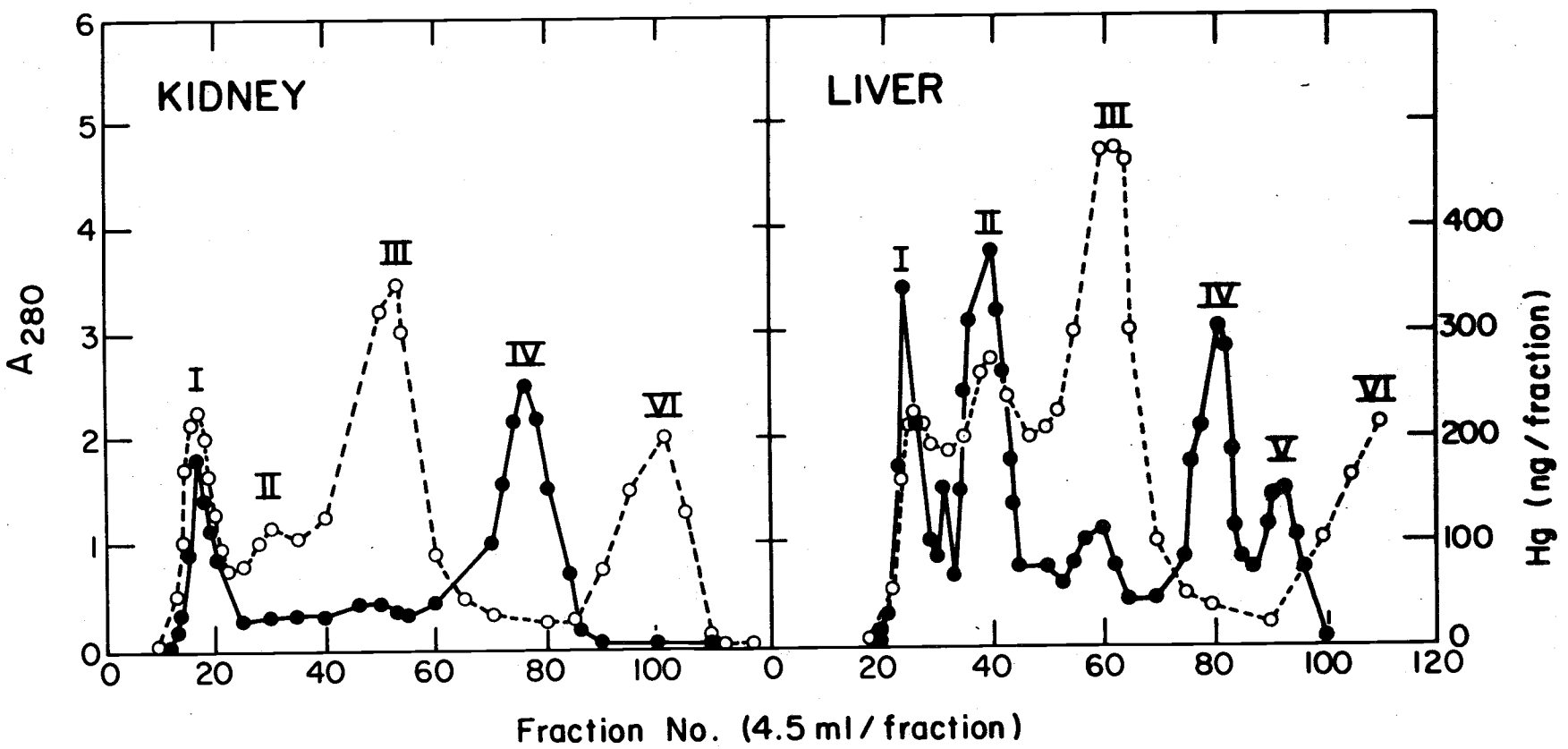
The elution profiles from G-75 columns of sea lion liver and kidney homogenate supernatants (Lee et al., 1976) displayed as much, sometimes more, mercury in the high MW fraction (void volume) than in the metallothionein region ($V_e/V_o \approx 2$). Metallothionein with its high content of SH groups binds mercury specifically and has been thought to play a role in mercury metabolism and detoxification.

The following question required consideration: are there one or more mercury binding proteins in the G-75 void volume fraction that have properties and a role similar to metallothionein? Or does this mercury bind to many proteins in a nonspecific way? One possibility is in the in vivo or in vitro formation of metallothionein polymers coupled by S-Hg-S bonds eluting in the void volume. These polymers may consist of a specific or favored number of subunits, or they may just build up randomly.

To search for high MW Hg-binding proteins, the supernatants of sea lion liver and kidney were chromatographed on Sephadex G-200 (Figure 12). A distinct protein peak (II) emerged between the G-200 void volume (I) and hemoglobin (III) peaks. Not much mercury was associated with this peak in the supernatant from kidney, but a major

Figure 12. Gel filtration on Sephadex G-200 of mercury binding proteins of the 48, 000 x g supernatant fractions from kidney and liver of sea lion No. 19. Mercury (●—●—●) and A_{280} (O---O---O).

Preparation of supernatants and column dimensions were as described under "Materials and Methods." Elution by gravity, flow rates between 16 and 20 ml/h.
Kidney: 4. 3 g, 13. 6 ml supernatant, 0. 1 M phosphate buffer, pH 7. 5.
Liver: 4. 9 g, 15. 2 ml supernatant, 0. 01 M Tris buffer, 0. 05 M NaCl, pH 8. 6.


KIDNEY
LIVER
I
II
III
IV
V
VI
A_{280}
Hg (ng / fraction)
Fraction No. (4.5 ml / fraction)
0
1
2
3
4
5
6
0
20
40
60
80
100
120
200
300
400

portion of the mercury in liver bound to it. Based on the 280 nm absorbancy, there appeared to be considerable protein eluting with the mercury in peak II as compared to metallothionein (peak IV). If mercury bound specifically to a small number of different protein species in peak II, obviously criteria other than separation on the basis of molecular weight had to be used to resolve these proteins.

The G-200 column was calibrated in order to estimate the MW of peak II. In numerous runs, V_e/V_o fluctuated between 1.5 and 1.6. It is thus safe to indicate a bracket of 120,000 to 180,000 daltons for the unknown mercury binding species (Figure 13). The average of V_e/V_o was 1.55, corresponding to 150,000 daltons.

Ion Exchange Chromatography

Fractions of the G-200 peak II were pooled and chromatographed on DEAE-cellulose, following a scheme previously employed by Kägi et al. (1974) in the purification of metallothionein. In a number of experiments, it was found that a protein containing relatively large amounts of mercury (Figure 14) was eluted at very low ionic strength (the conductivity was between 1 and 2 mmho) immediately after the buffer concentration had begun to rise. A flat gradient (1 mM to 100 mM buffer) was employed in the beginning to widely spread the loosely binding proteins. Preliminary experiments had shown that the proteins eluting at higher ionic strength did not bind any significant

Figure 13. Calibration curve of the G-200 column. The shaded area refers to the unknown mercury binding protein in peak II.

Column dimensions are described under "Materials and Methods." Eluting buffer was 20 mM phosphate, pH 7.5.

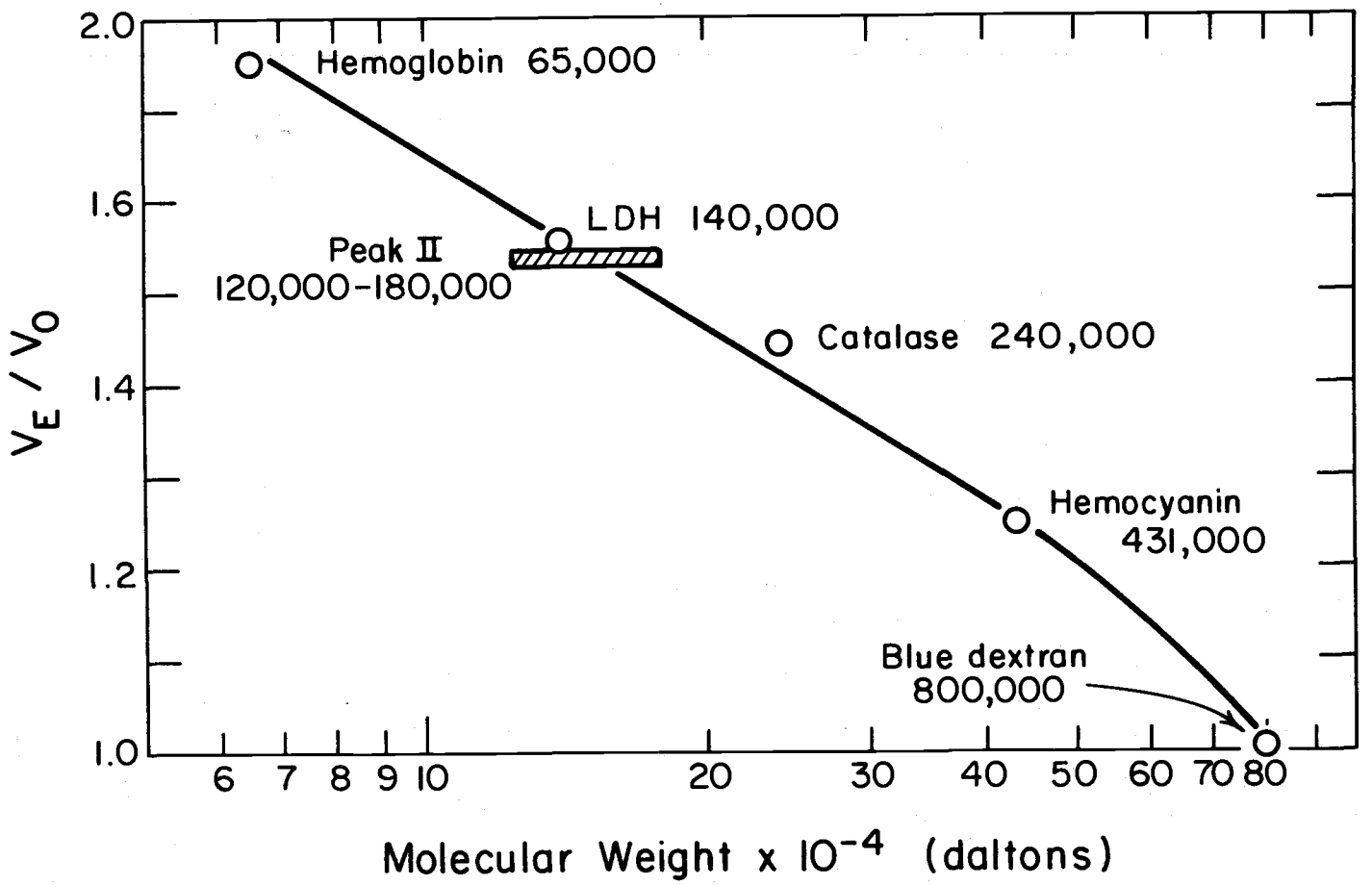
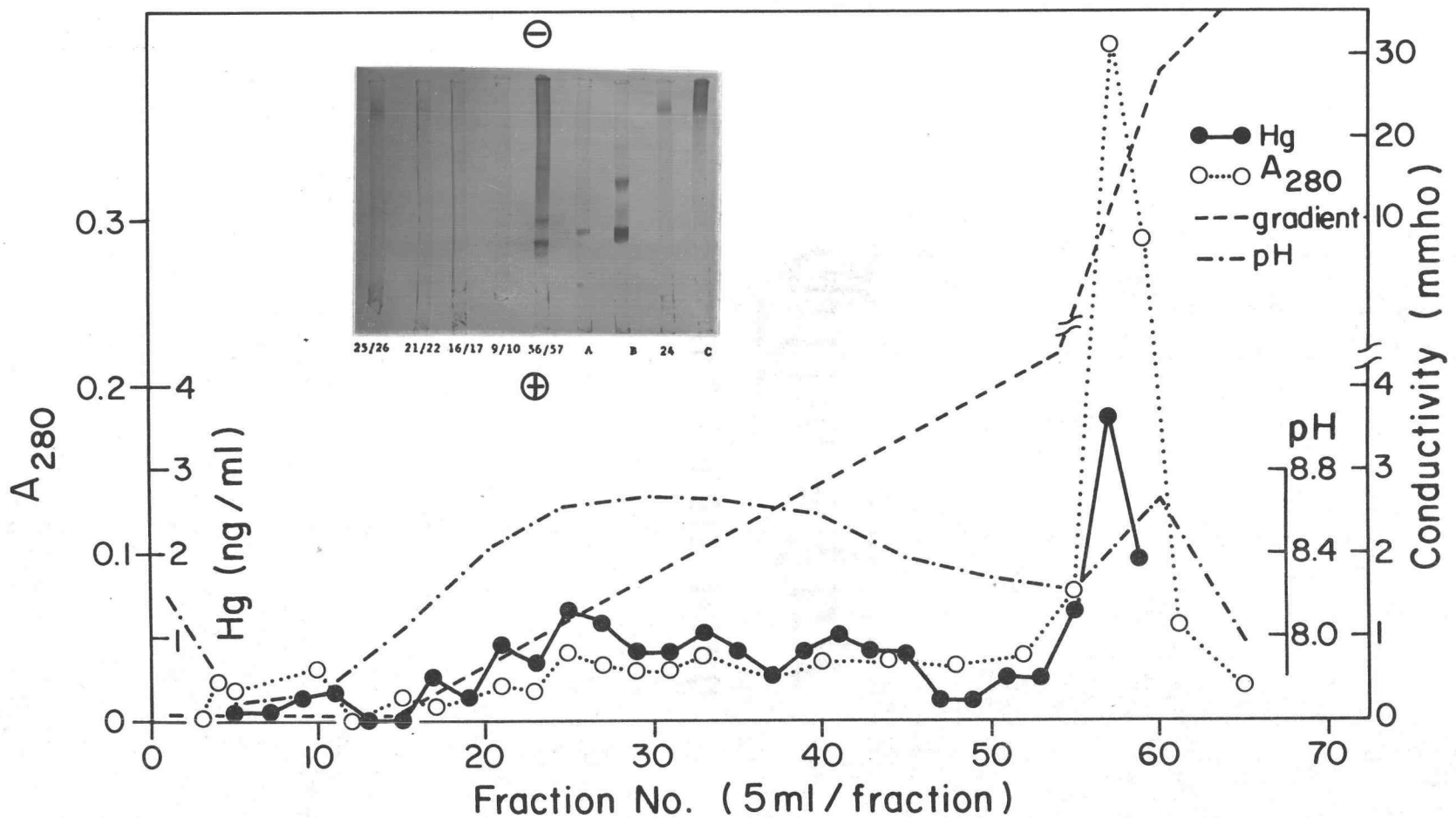

2.0
1.6
1.4
1.2
1.0
V_E / V_O
Hemoglobin 65,000
LDH 140,000
Peak II
120,000-180,000
Catalase 240,000
Hemocyanin
431,000
Blue dextran
800,000
6 7 8 9 10 20 30 40 50 60 70 80
Molecular Weight x 10^{-4} (daltons)

Figure 14. Peak II (from the G-200 column of sea lion No. 19 liver supernatant) chromatographed on DEAE-cellulose. Mercury (●——●——●); A_{280} (O·····O·····O); conductivity (- - - - -); and pH (— · — · —). Bed volume 30 ml; phosphate-borate buffer, pH 8.2 containing 0.1 mM DTE and buffer concentration from 1 mM to 100 mM.

Results of gel electrophoresis of some of the column fractions are shown in the inset. Migration from cathode to anode. The numbers below the gels refer to the respective column fractions that were electrophoresced in the gel. Gels A and B contained BSA and C contained a fraction of another DEAE-column as explained in the text. Some of the gels were fractured in the procedure of removing them from the tubes, but no stained bands were affected.


⊖
⊕
25/26 21/22 16/17 9/10 56/57 A B 24 C
Hg
A280
gradient
pH
A280
Hg (ng/ml)
pH
Conductivity (mmho)
Fraction No. (5ml/fraction)
0
10
20
30
40
50
60
70
0.3
0.2
0.1
0
1
2
3
4
8.8
8.4
8.0

amount of mercury. Therefore, after the slightly binding material had been separated, the column was washed with 2 M NaCl resulting in the elution of material exhibiting high absorption at 280 nm combined with a small amount of mercury at the end of the elution profile. The column was equilibrated and eluted at pH 8. 2. Due to the low concentration of buffer (1 mM), the pH fluctuated considerably during the run.

Gel electrophoresis (Davis, 1964; alkaline medium, direction of migration from cathode to anode) of some of the column fractions is shown on the inset photograph (Figure 14). The material from the first peaks (fractions 9/10, not binding to the column, and fractions 16/16 slightly binding to the column) was not visible in the gel after staining with amido schwarz. To make sure that there was not any positively charged material in fractions 9/10 and 16/17, the electrophoresis was repeated under identical conditions, but with reverse polarity. This time no bands were detected in any of the gels after staining with amido schwarz.

A weak band was found after electrophoresis of fractions 21/22, and fractions 24 and 25/26 produced clearly visible bands, all three with an R_f value of 0. 12. Fractions 56/57 contained a great many proteins, not all of them well separated, as seen in the corresponding gel.

Gels A and B contained BSA standards to quantitate the amount

of protein. A sample from the mercury binding peak of one of the preliminary DEAE-columns was electrophoresced in gel C. This column had been equilibrated with 5 mM buffer as opposed to 1 mM in the column depicted in Figure 14. Presumably, the higher buffer concentration prevented the protein that contained mercury from binding to the DEAE-cellulose. Instead, this protein eluted with the non-binding fraction. Therefore, gel C displayed a band of an R_f value of 0.12 but also unseparated material of lower R_f values (lower negative charge, ergo not binding to DEAE-cellulose at pH 8.2).

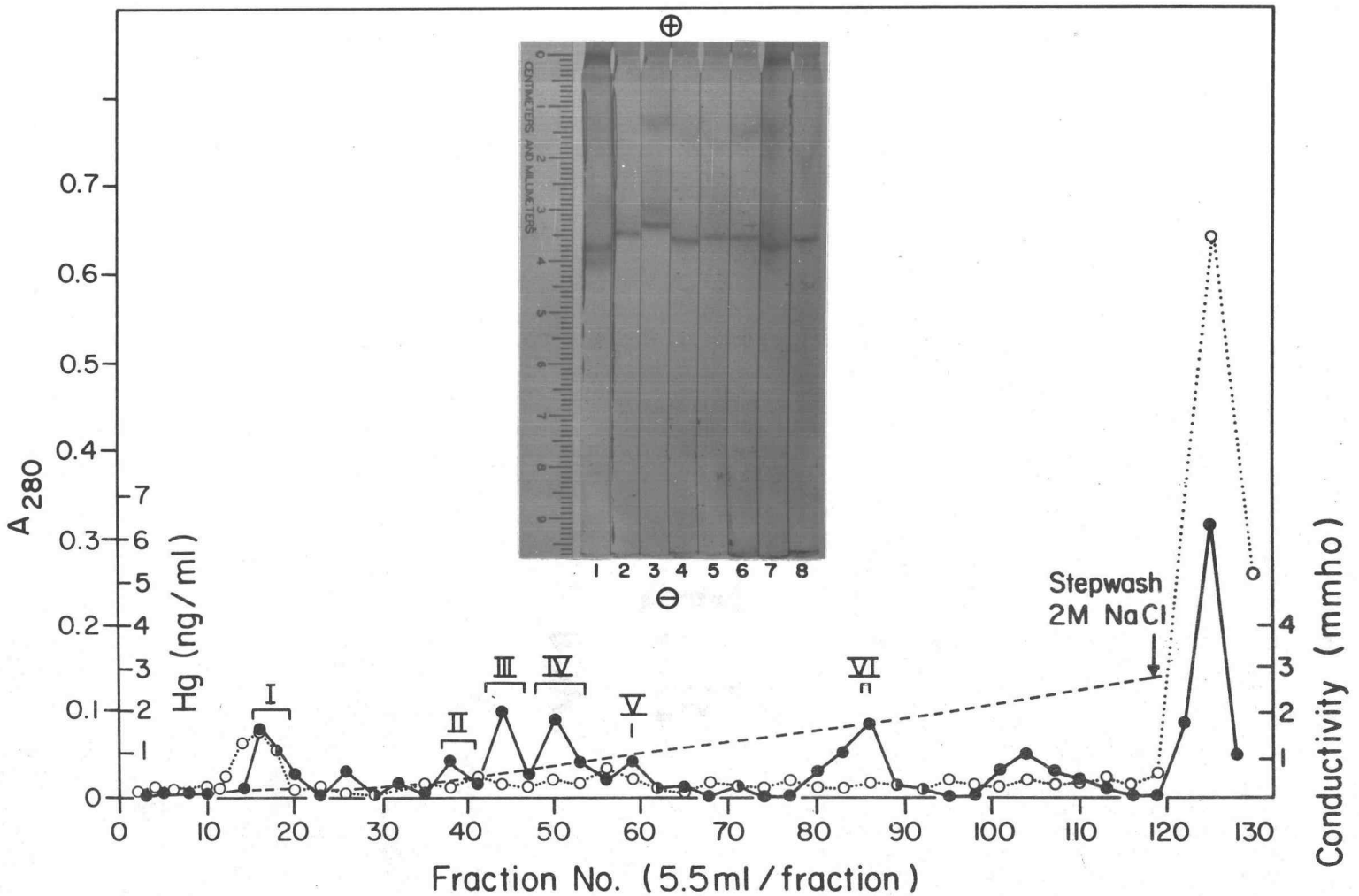
Based on the assumption of a MW of around 150,000, the stoichiometric ratio protein:mercury in the gel bands was calculated to 270:1. The same calculation for the material of fractions 25/26 yielded a ratio of 40:1 prior to electrophoresis. This indicated that mercury must have been lost during electrophoresis. But loss of mercury already occurred one step prior to this since recovery of mercury from the various ion exchange columns was only 50-70%.

Another trial with DEAE-cellulose (120 ml bed volume) is shown in Figure 15. A flat, linear NaCl-gradient (0 to 50 mM in 500 ml) in the same buffer, 1 mM phosphate-borate (Kolthoff, 1925), pH 8.2 containing 0.1 mM DTE eluted the slightly binding mercury-rich material and separated it from non-binding material.

By use of a different buffer system for the gel electrophoresis (pH 2.3, direction of migration from anode to cathode--Brewer and

Figure 15. Peak II (from the G-200 column of sea lion No. 19 liver supernatant) chromatographed on DEAE-cellulose. Mercury (●——●——●); A_{280} (O····O····O); and conductivity (- - - - -). Bed volume 120 ml; and 1 mM phosphate-borate buffer, pH 8. 2, containing 0. 1 mM DTE. The protein was eluted with a linear NaCl gradient from 0 to 50 mM. Stronger binding materials were eluted with 2 M NaCl in the same buffer.

Fractions that were concentrated and electrophoresced are numbered with roman numerals. A photograph of the gels is shown in the same figure. Fraction I was electrophoresced in gel 1, fraction II in gel 2, etc. A mixture of fractions I, II, and III was electrophoresced in gel 7; a mixture of fractions IV, V, and VI in gel 8.


⊕
⊖
1 2 3 4 5 6 7 8
CENTIMETERS AND MILLIMETERS
A280
Hg (ng/ml)
Conductivity (mmho)
Fraction No. (5.5ml/fraction)
Stepwash
2M NaCl
I
II
III
IV
V
VI
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
80
90
100
110
120
130

Ashworth, 1969), thinner protein bands were obtained. Due to reversal of net charge of the proteins as well as polarity of the electrodes, the non-binding proteins (peak I, gel 1) moved further than the binding proteins. The proteins from fractions IV, V, and VI were electrophoresced in gels with corresponding arabic numerals and as a mixture in gel 8. They displayed identical electrophoretic mobilities in this system. Gel 7 contained a mixture of fractions I, II, and III. These proteins showed slightly different R_f values when run in gels 1, 2, and 3, and a smeared band in gel 7. This result suggests that fractions I, II, and III consist of different protein species, whereas the same or similar proteins are found in fractions IV, V, and VI. As observed previously, mercury analysis showed only very low levels to be present.

Obviously this scheme of separation (G-200, DEAE-cellulose, gel electrophoresis) was not applicable for two reasons:

1) Loss of mercury during DEAE-cellulose chromatography (other ion exchangers, for cations as well as naions, had been tried with no better success) and polyacrylamide disk electrophoresis. After losses of mercury during centrifugation, gel filtration, ion exchange chromatography, and concentration in dialysis bags, the mercury levels had reached a threshold (1 to 2 ng/ml) such that any further handling, e. g. electrophoresis and solubilization of the gel slices, brought them close to the detection limit of atomic absorption

analysis.

2) The amounts of material that could be processed were too small for a multistep purification procedure.

Specific Ion Mediated Lipophilic Chromatography

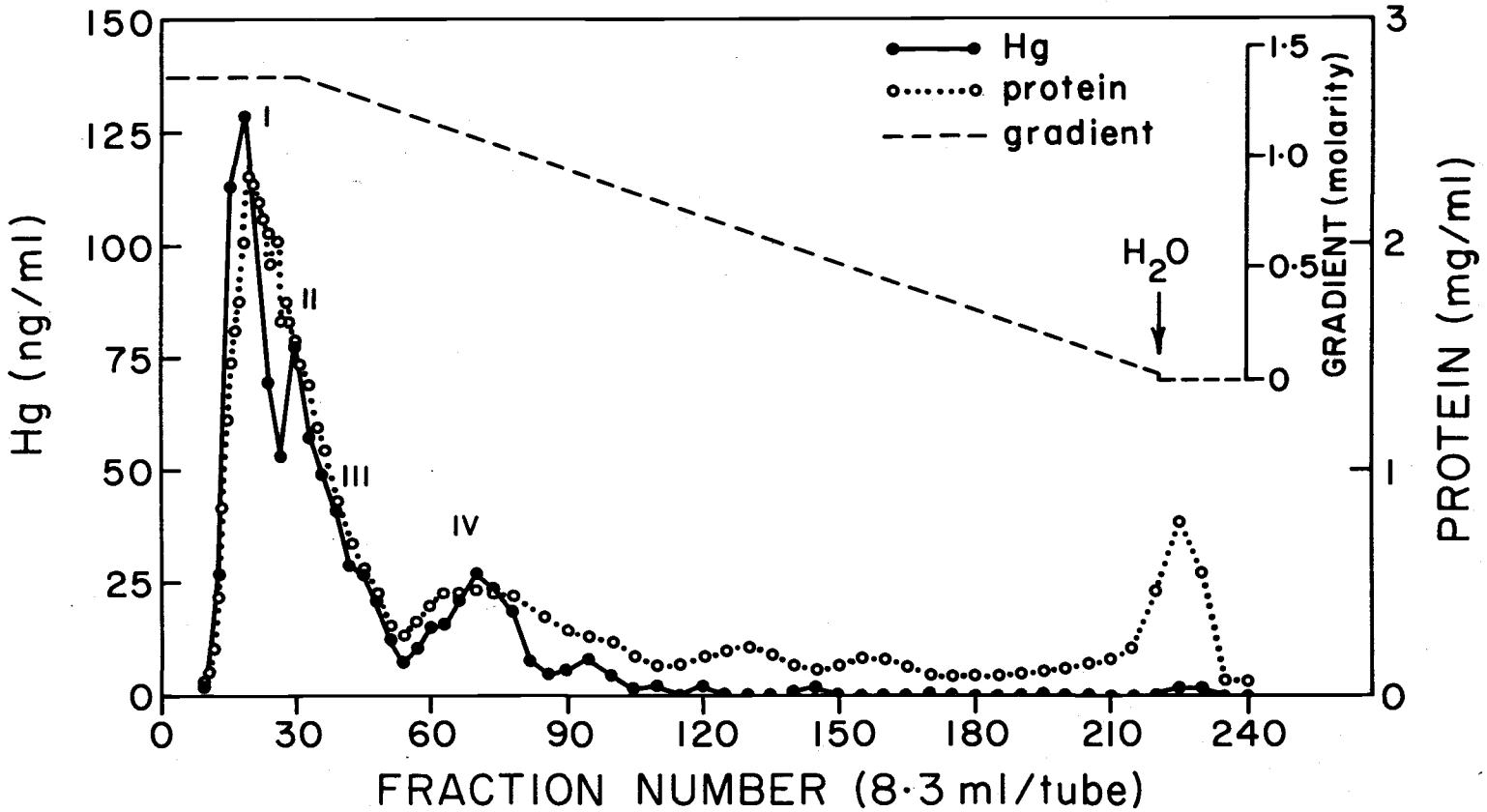
A procedure that would handle bulk quantities of protein and that did not involve a matrix with such a high affinity for mercury had to be found.

Ammonium sulfate fractionation permitted the separation of a mercury-rich fraction of sea lion liver supernatant (30 to 50% saturation of ammonium sulfate at 0° C in 0. 2 M phosphate buffer, pH 7. 5) from metallothionein and hemoglobin.

Preliminary experiments with specific ion mediated lipophilic chromatography (Rimerman and Hatfield, 1973) gave 100% recovery of mercury, and since this method is based on absorption due to hydrophobic binding, larger amounts of protein (10 mg protein per ml of bed volume) could be accommodated than be gel filtration chromatography. One experiment with such a column is shown in Figure 16. A column packed with a bed of 90 ml of 4B-Sepharose with covalently attached norleucine (see "Materials and Methods" section for presumed chemical structure) was equilibrated with 0. 2 M phosphate buffer, 30% saturated with ammonium sulfate. The 30 to 50% fraction of the supernatant was applied and eluted with a linear

Figure 16. Specific ion mediated lipophilic chromatography of sea lion liver cytosol fraction. Mercury (●——●——●); protein measured according to Mejbaum-Katzenellenbogen (1955) (O····· O····· O); and molarity gradient (- - - - -).

The 30 to 50% ammonium sulfate fraction of sea lion No. 19 liver 48,000 x g supernatant (60 ml = 18.5 g liver) was eluted from a 90 ml bed of 4B-Sepharose with covalently attached DL-norleucine, preequilibrated with 0.2 M phosphate buffer, 30% saturated with ammonium sulfate at 4° C. The eluting gradient (1570 ml) was linear from 1.37 M to 0.02 M. Finally, the column was washed with redistilled water.


Hg
protein
gradient
I
II
III
IV
H_2O
Hg (ng/ml)
PROTEIN (mg/ml)
GRADIENT (molarity)
FRACTION NUMBER (8·3 ml/tube)
0
25
50
75
100
125
150
30
60
90
120
150
180
210
240
1
2
3
0·5
1·0
1·5

molarity gradient decreasing from 1. 37 M to 0. 02 M.

The mercury was associated with essentially 3 peaks: the non-binding material (I), the slightly binding material (II), and peak IV. These peaks and fraction III, the downward slope of peak II, were chromatographed on G-200 to obtain their MW-distribution. The representation (Figure 17) looks very disperse. Each fraction (I, II, III, and IV) contained material of a relative elution volume (V_e/V_o) around 1. 5 to 1. 6. But the low ratios of ng Hg/mg protein in this region do not indicate specific binding. A ratio of 1, 330 ng Hg/mg protein would indicate a 1:1 stoichiometry. But generally there is 10 times less mercury per protein even after three purification steps with an estimated overall purification of 30-fold.

To confirm these results of successive ammonium sulfate precipitation, specific ion mediated lipophilic chromatography, and gel filtration, the order of the latter two steps was reversed. The fraction precipitated by 50%, but soluble in 30% ammonium sulfate was run through a G-200 column. Three different batches of hydrophobic 4B-Sepharose were prepared: valine was attached to one, norleucine to another, and α-amino caprylic acid to the third. The 150, 000 dalton fraction was made 0. 2 M in phosphate and 30% saturated with ammonium sulfate, and aliquots chromatographed, one on each batch of hydrophobic Sepharose (Figure 18). The proteins were eluted stepwise by decreasing ammonium sulfate concentration. Each

Figure 17. Gel filtration on Sephadex G-200 of fractions I to IV from the norleucine-Sepharose column. Mercury (●——●——●); and protein (O- - -O- - -O), measured by the method of Mejbaum-Katzenellenbogen (1955). Ratios of ng Hg/mg protein are indicated above the respective peaks, relative elution volumes in parentheses below.

The four fractions were chromatographed one after another on a G-200 column of 90 cm length and 2.5 cm inner diameter. Sample sizes were 10 to 15 ml, elution buffer was 20 mM phosphate.

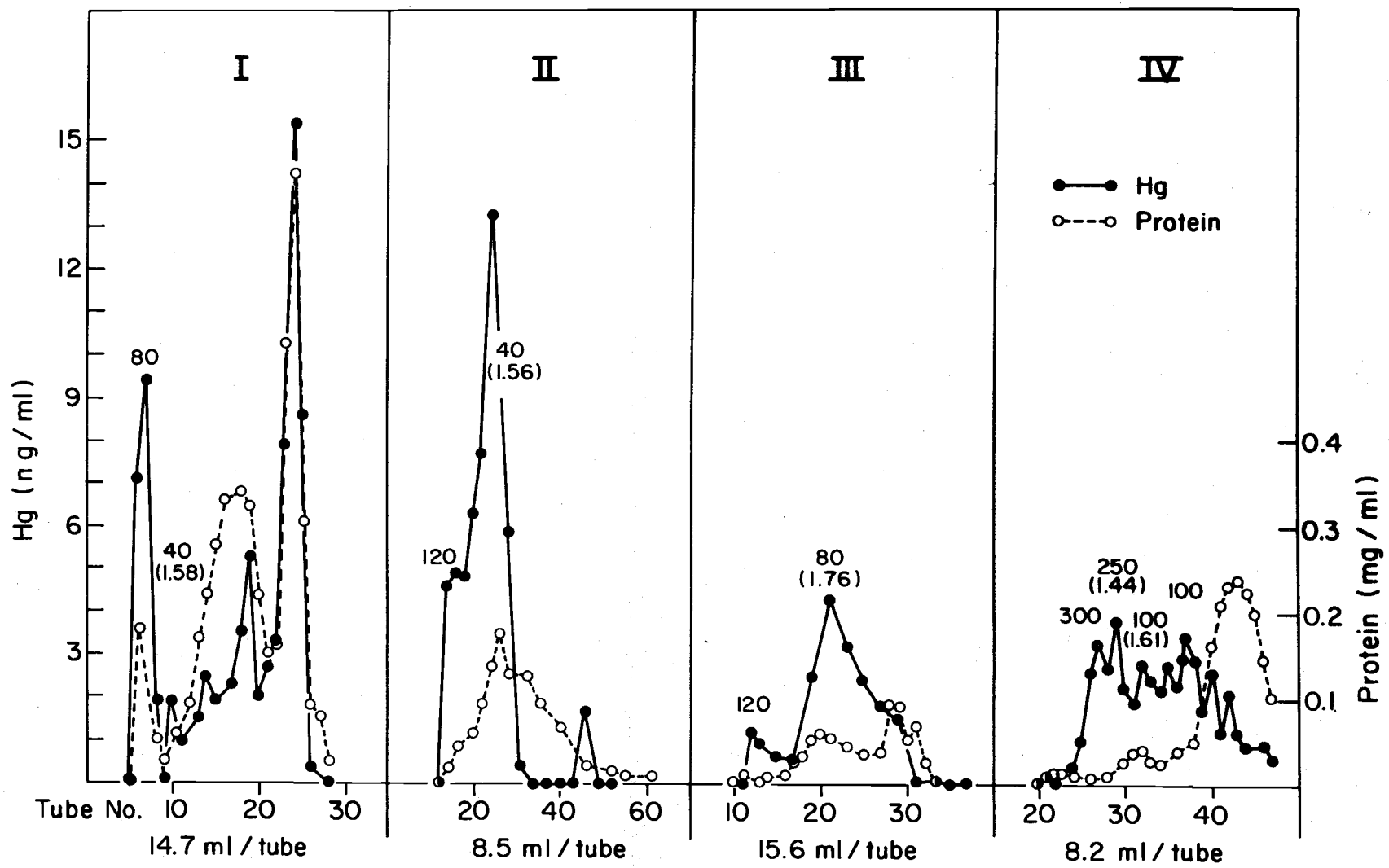
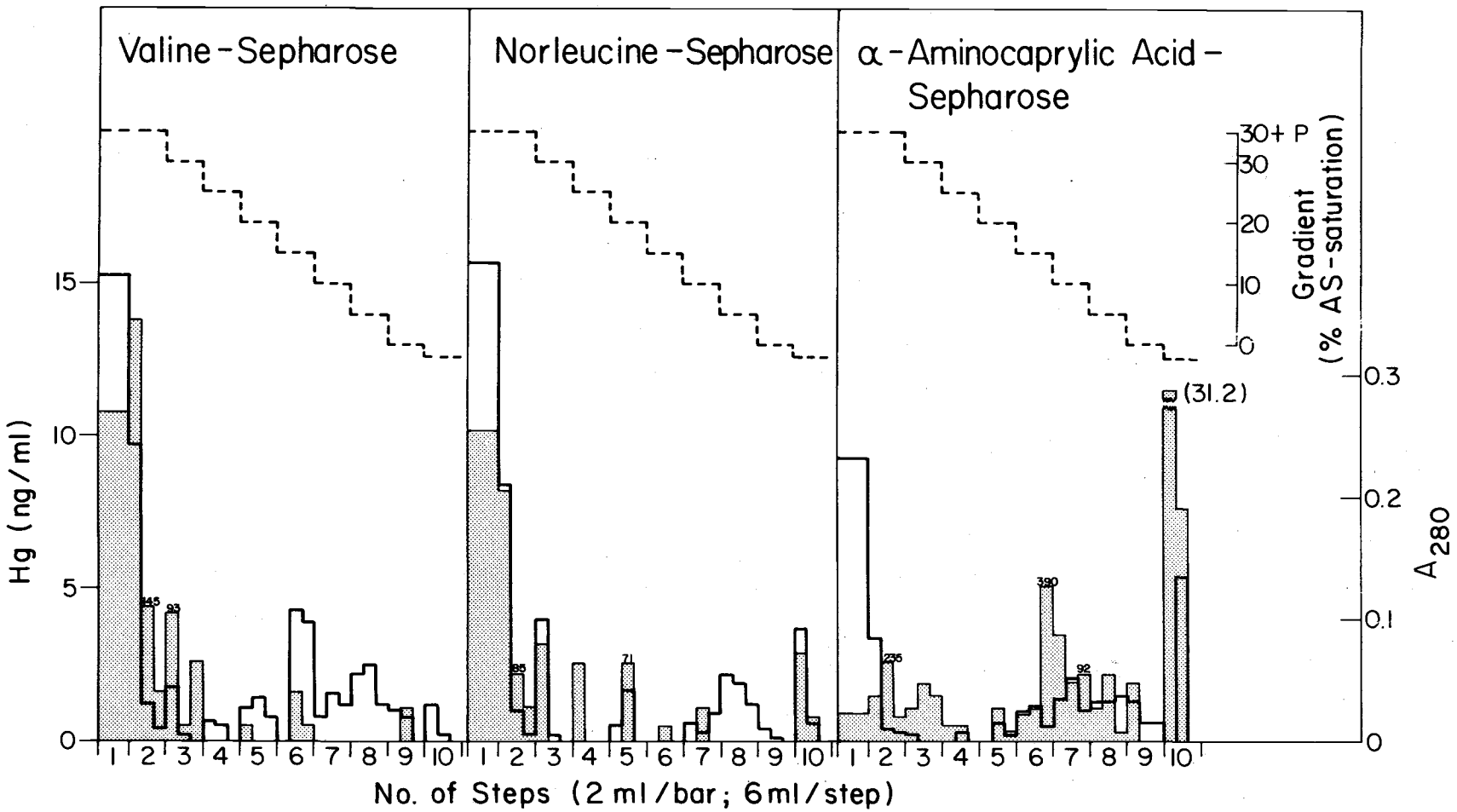

I
II
III
IV
Hg
Protein
Hg (ng/ml)
Protein (mg/ml)
80
40
(1.58)
40
(1.56)
120
80
(1.76)
120
250
(1.44)
100
300
100
(1.61)
Tube No.
14.7 ml / tube
8.5 ml / tube
15.6 ml / tube
8.2 ml / tube

Figure 18. Chromatography of the 150,000 dalton fraction of sea lion No. 19 liver supernatant in media of different hydrophobicity. Mercury is represented as shaded bars and A_{280} as open bars with wider boundaries. Ratios of ng Hg/A_{280} are indicated above the mercury-rich fractions. The gradient is represented by a dashed line.

Bed volume 1 ml each, and sample volume 5 ml each. The same stepwise gradient from 30% ammonium sulfate plus 0.2 M phosphate to zero molarity followed by 0.01 N NaOH eluted all three columns. Each step consisted of three washes with 2 ml of eluant.


Valine-Sepharose
Norleucine-Sepharose
α-Aminocaprylic Acid-Sepharose
Hg (ng/ml)
15
10
5
0
30+ P
30
20
10
0
Gradient (% AS-saturation)
0.3
0.2
0.1
0
A280
(31.2)
145
95
85
71
226
390
92
1 2 3 4 5 6 7 8 9 10
No. of Steps (2 ml/bar; 6ml/step)

step consisted of three washes with a solution that was 5% less saturated than in the previous step. The first step of washings was performed with 0. 2 M phosphate buffer, the following steps in 0. 02 M phosphate. The last step consisted of three rinses with 0. 01 N NaOH.

Comparing all three elution profiles, one finds that with increasing hydrophobic properties of the Sepharose, more protein (and mercury) bound to the matrix and was eluted later (at lower ammonium sulfate concentrations). The valine substituted Sepharose bound only two major mercury containing fractions. They were eluted at 30% ammonium sulfate in 0. 2 M and 0. 02 M phosphate, respectively. As the matrix became more hydrophobic (norleucine), four mercury containing fractions bound to the support eluting at 30, 25, and 20% ammonium sulfate and 0. 01 N NaOH, respectively. In a medium as hydrophobic as α-aminocaprylic acid, hardly any mercury passed through the column unbound, and the major mercury peaks were eluted with 15 and 10% ammonium sulfate and 0. 01 N NaOH (most of the mercury). The recovery of mercury from the three columns was 115%, 79. 5%, and 90. 7%, respectively.

An identical experiment was conducted with the void volume fraction of the G-200 column. These elution profiles are not shown because no mercury binding proteins could be resolved (they either did not bind to the column as in the case of valine- and norleucine-Sepharose, or as with α-aminocaprylic acid, they bound too tightly

and could only be eluted with NaOH).

The purification (in terms of a mercury:protein ratio) achieved with this attempt was no greater than before (Figure 17), and again it was shown that mercury was distributed more or less evenly over most of the proteins.

The question posed in the beginning: "is mercury binding specifically to one, several or many proteins of 150,000 daltons?" was thus resolved by these findings. There was no protein of high MW which specifically bound mercury to a similar extent as does metallothionein, and which would have warranted further efforts aimed at its isolation and purification. Moreover, no support was gained for large amounts of a hypothetical mercury containing polymetallothionein of around 150,000 daltons. Excessive amounts of mercury were rather found to be bound to many different proteins of high molecular weight.

Estimation of Metallothionein Content

Metallothionein concentrations in livers and kidneys of three bearded seals, three sea lions, and three walrus were determined by the method of Piotrowski et al. (1973a). The metallothionein content of marine mammal liver (Table V) was in the same range as the 0.1 mg/g tissue reported by Piotrowski et al. for normal rats. In marine mammal kidney it did not quite reach the value reported by Piotrowski et al. for rat kidney (0.4 mg/g). The average

metallothionein level in marine mammal kidneys (0. 112 mg/g) was higher than that found in livers of the same species (0. 093 mg/g) but the difference is not significant at the 95% probability level.

TABLE V. METALLOTHIONEIN AND HEAVY METAL CONTENT IN MARINE MAMMAL LIVERS AND KIDNEYS

		Metallothionein (mg/g tissue)	Total (Cd+Hg) (nmol/g tissue)	Soluble (Cd+Hg) (nmol/g tissue)
Liver				
Bearded Seal	No.			
	1	0. 110	59	30
	2	0. 041	59. 5	35
	4	0. 061	220	111
Walrus	No.			
	2	0. 041	74	89
	4	0. 163	87. 5	97
	5	0. 044	80	54
Sea Lion	No.			
	19	0. 100	218	146
	20	0. 092	281	202
	21	0. 215	612	--
Kidney				
Bearded Seal	No.			
	1	0. 100	218	146
	2	0. 092	281	202
	4	0. 112	591	326
Walrus	No.			
	2	0. 125	123	124
	4	0. 215	649	381
	5	0. 109	625	301
Sea Lion	No.			
	19	0. 056	180	100
	20	0. 074	82	67
	21	0. 128	57	--

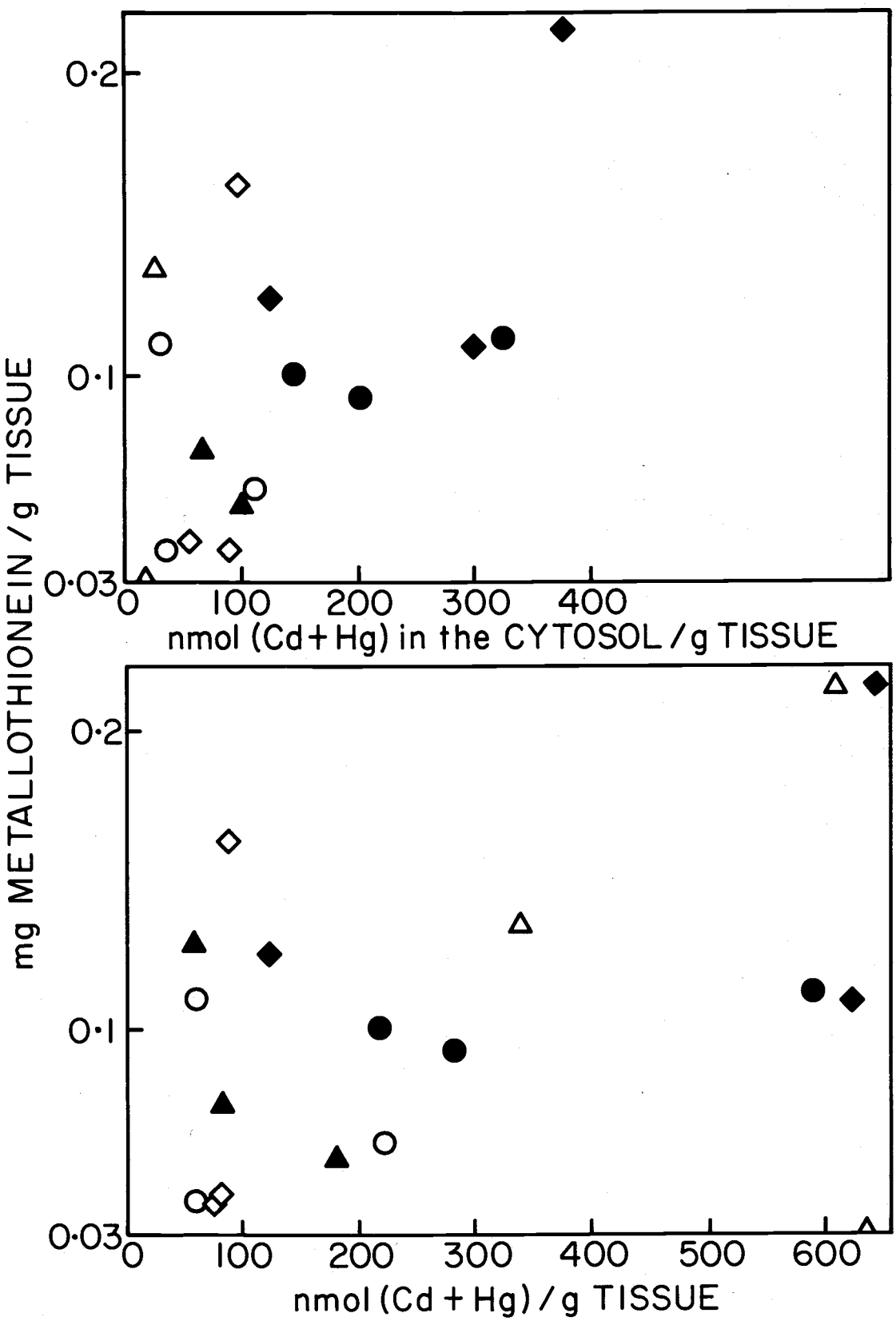
There was no good correlation of metallothionein content with heavy metal concentration (see data in APPENDIX I) in the livers or kidneys of any one marine mammal species. The sample sizes were too small, and the errors probably too large. Only if livers and kidneys of all three species are viewed together, can a very limited linear correlation be seen (Figure 19). It is best for cadmium and cadmium plus mercury (on a molar basis) in the cytosol of kidney cells (R^2=0. 5561 and 0. 5456, respectively; Table VI). The R^2-value of 0. 3797 for metallothionein with mercury in kidney supernatant is derived from a negative R (=-0. 6162), indicating that metallothionein levels decrease with increasing mercury concentrations.

TABLE VI. CORRELATION COEFFICIENTS (R^2) BETWEEN METALLOTHIONEIN LEVEL AND HEAVY METAL CONCENTRATION IN MARINE MAMMAL LIVER AND KIDNEY[a]

Fraction and tissue	Correlation coefficients (R^2)		
	Cd	Hg	Cd + Hg
Whole cell			
Liver	0. 0822	0. 1092	0. 0902
Kidney	0. 1515	0. 0049	0. 1548
Liver and kidney	0. 0881	0. 0226	0. 1591
Cytosol			
Liver	0. 0092	0. 0363	0. 0142
Kidney	0. 5561	0. 3797	0. 5456
Liver and kidney	0. 3323	0. 0680	0. 3331

[a]Linear correlation determined by computer OS-3 using SIPS (statistical interactive programming system).

Figure 19. Relationship between metallothionein and heavy metal contents in marine mammal livers and kidneys. Liver and kidney, respectively: walrus (◇, ◆); bearded seal (O, ●); sea lion (△, ▲).


0·2
0·1
0·03
0
100
200
300
400
nmol (Cd+Hg) in the CYTOSOL / g TISSUE
mg METALLOTHIONEIN / g TISSUE
0·2
0·1
0·03
0
100
200
300
400
500
600
nmol (Cd + Hg) / g TISSUE

The low degree of linear correlation between metallothionein and metal concentrations was not expected. It may reflect the accurate picture of the situation, or it may be due to errors in the analysis, or to the inapplicability of Piotrowski's method to marine mammals.

Tracing Possible Errors in the Metallothionein Determination

To find out whether only metallothionein was present in the counted solutions in the Piotrowski et al. (1973a) assay, aliquots of some of the TCA-supernatants were passed through a Sephadex G-50 column and dialyzed against 50 mM ammonium sulfate buffer (pH 8.5). Special small pore dialysis tubing with a cut-off point at MW 3,500 was filled with aliquots of the TCA-soluble fraction of walrus No. 5 kidney and bromophenol blue (MW=670) as marker substance. After 24 h of dialysis with three changes of buffer, almost all the bromophenol blue had diffused out of the dialysis bag. The radioactivity of ^{203}Hg bound to protein in the bag, however, was unchanged. This result indicates that no low MW form of mercury (such as $^{203}Hg^{2+}$) remained in the TCA-soluble fraction.

Figure 20 shows gel filtration of the TCA-soluble fraction of bearded seal No. 2 kidney with and without addition of 0.1 mg horse kidney metallothionein. Three radioactive peaks appeared at relative elution volumes of 1.7-1.8, 2.1, and 3.6-3.7, respectively. No other

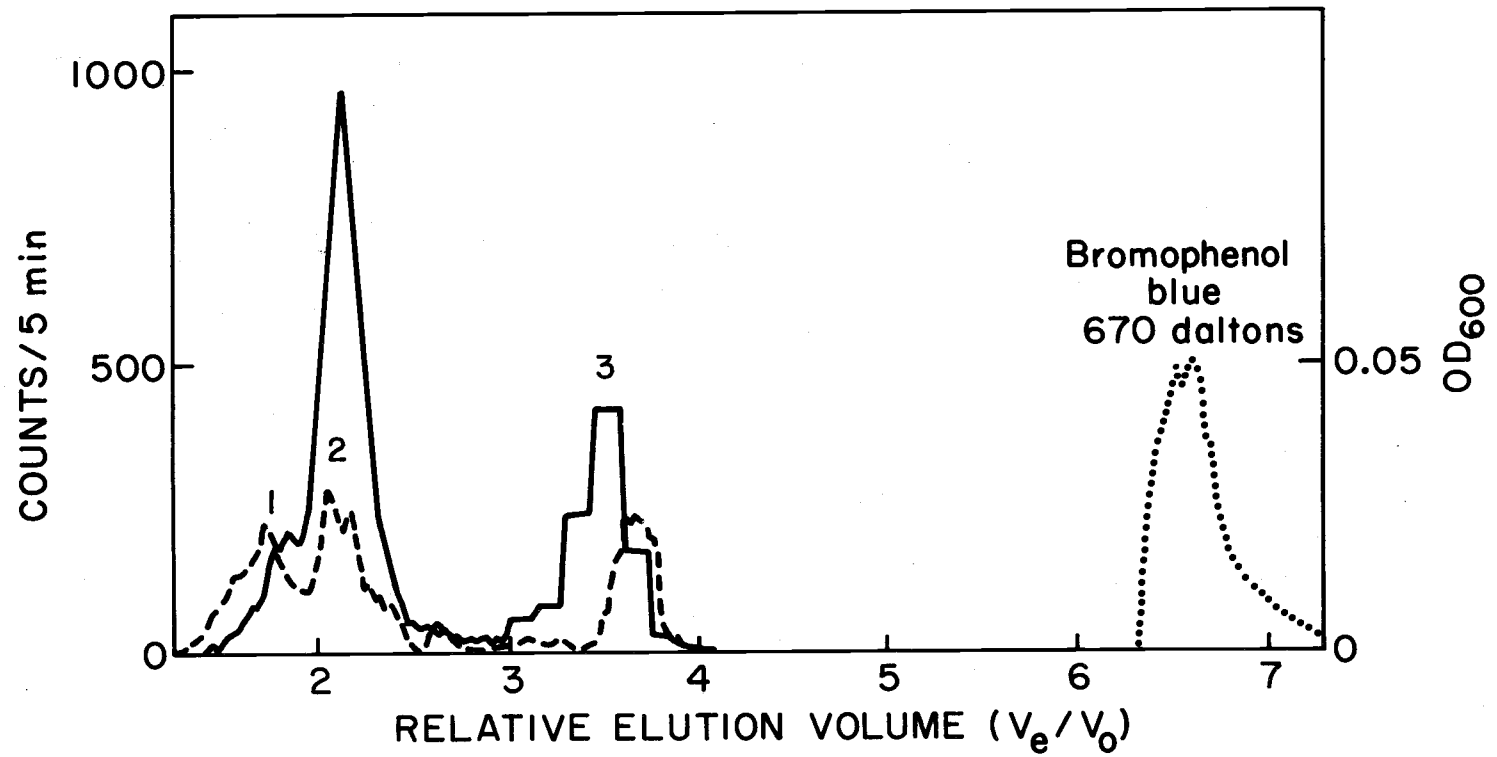


Figure 20. Sephadex G-50 gel filtration of the TCA-soluble fraction of bearded seal No. 2 kidney after adding $^{203}HgCl_2$. Horse kidney metallothionein was added to one sample (solid line) and the other sample was chromatographed without metallothionein standard (dashed line). The dotted line shows the relative elution volume of bromophenol blue that was added to the second sample.
Column dimensions: length 50 cm and inner diameter 2. 5 cm. Eluting buffer 50 mM ammonium sulfate, pH 8. 5.

radioactivity was detected in the effluent. Bromophenol blue, added as marker substance, displayed a relative elution volume of 6. 75, proving that no compound of a MW lower than at least 1, 000 bound any $^{203}Hg^{2+}$. Addition of horse kidney metallothionein as an internal standard caused a substantial increase in peaks 2 and 3 but no change in peak 1.

These results suggest that both peaks 2 and 3 were derived from metallothionein. Prior to gel filtration, the samples had been stored in solution at room temperature for one week. This long period of storage and availability of oxygen may have caused profound changes in the molecular size of metallothionein. Aggregation of two molecules to form a dimer is one possibility, breakdown another. The MW reported for marine mammal metallothionein ranges from 7, 000 to 10, 000 daltons (Lee and Buhler, 1974; and Olafson and Thompson, 1974). From Figure 20 it is apparent that this MW does not differ markedly from the MW of horse kidney metallothionein. Since the horse kidney metallothionein had been an electrophoretically homogeneous substance before the experiments described here were initiated,[7] it can be concluded that bearded seal as well as horse kidney metallothioneins undergo the same changes (aggregation or breakdown) upon storage in the open air at room temperature.

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[7]According to information by Dr. P. D. Whanger (Dept. of Ag. Chem., OSU) in whose laboratory the sample had been prepared.

The G-50 column was not calibrated, only the void volume was known and the relative elution volume of bromophenol blue. Under slightly different conditions (same column dimensions, but 20 mM phosphate buffer, pH 7.5) a sample of sea lion No. 19 kidney metallothionein was found to display a relative elution volume of 1.9 in a Sephadex G-50 column. It is therefore likely that peak 2 (V_e/V_o = 2.1) corresponded to the original form of metallothionein and peak 3 (V_e/V_o = 3.6 - 3.7) was a breakdown product. Peak 1 (V_e/V_o = 1.7 - 1.8) may have constituted a high MW form of metallothionein only characteristic of marine mammals, or it may have been a contaminating substance present in bearded seal kidney of higher MW than metallothionein that also bound $^{203}Hg^{2+}$.

The relative distribution of radioactivity differed between the two runs. In the unspiked sample it was 36 and 31% for peak 2 and 3, respectively, and in the sample spiked with horse metallothionein, 57 and 34%.

The G-50 column chromatography (Figure 20) and the dialysis experiment suggest that no compounds smaller than metallothionein interfere with the assay in walrus and bearded seal kidney, but contamination might be present at a MW higher than 10,000.

Because of possible errors which would render Piotrowski's method inapplicable to marine mammals, the lack of correlation of metallothionein content with heavy metal concentrations does not

prove that metallothionein synthesis is not induced in kidney and liver by naturally occurring levels of cadmium and mercury, although this is suggested by the data in Figure 19 and Table VI. Only cadmium levels in the cytosol of kidney cells were reasonably correlated with the amounts of metallothionein found in he same fraction.

RESULTS. PART III: THE DEMETHYLATION OF METHYLMERCURY IN SEA LION TISSUES--*IN VITRO* APPROACH

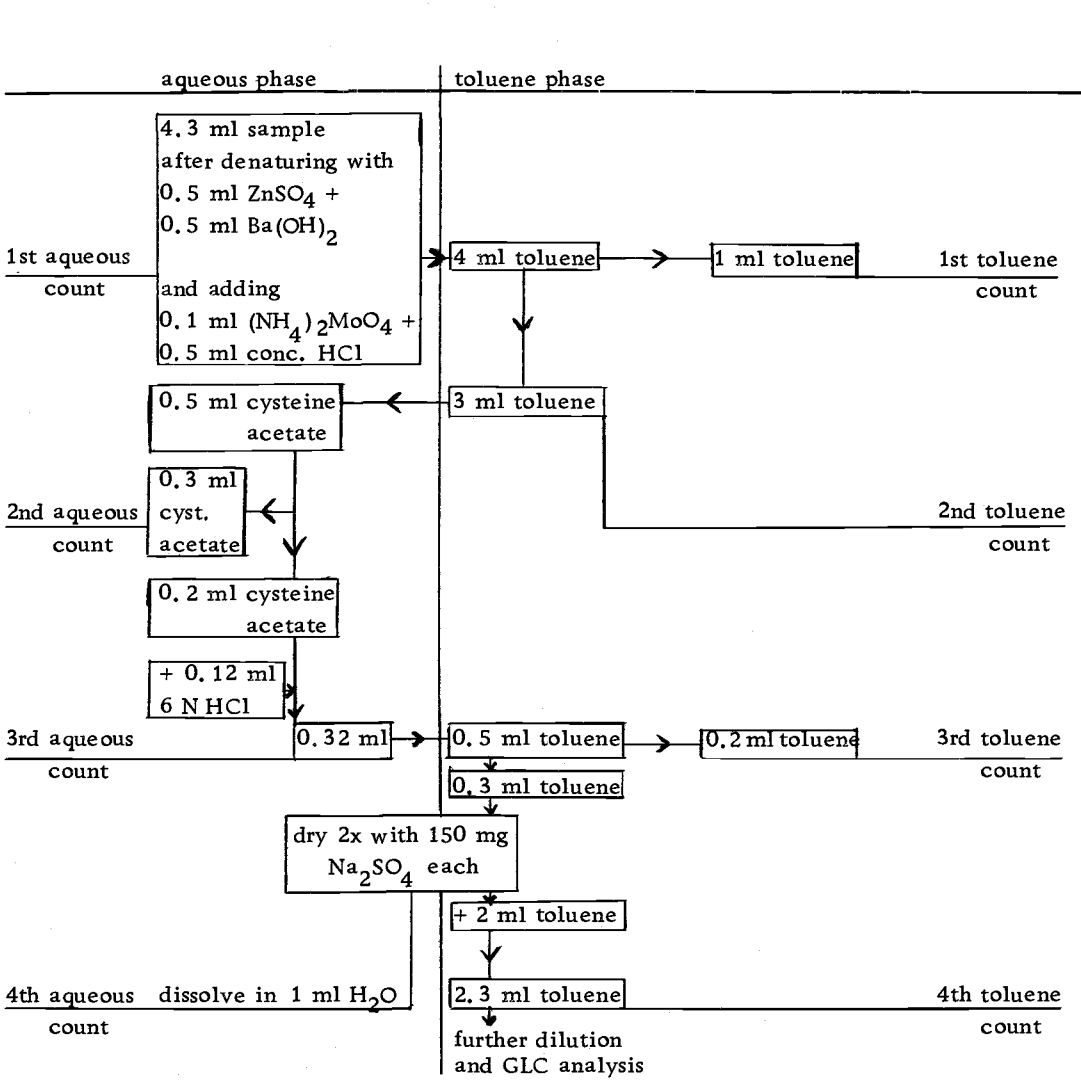
No demethylation of methylmercury was found *in vitro* with rat liver and kidney. This confirms results published elsewhere (Fang and Fallin, 1974) and most likely many unpublished data. The rat tissues had been prepared by using a Potter-Elvehjem homogenizer as described above.

A control experiment (incubation of 0.33 μg Hg/ml as MeHg at 37° C in a 0.017 M cysteine solution, pH 2) revealed that inorganic mercury was liberated at a rate of 4% per day with NaClO as antibacterial agent and of 17% per day with a mixture of antibiotics (see "Materials and Methods"). The experiment was conducted over a period of 15 days and the rates were established by linear extrapolation of semilogarithmic plots of the data. A pH of 2 may seem too low compared with physiological values, but even higher hydrogen ion concentrations are encountered in the stomach. The purpose of this experiment was to establish the maximal demethylation rate under reasonable inorganic conditions in order to be able to compare it with the rate of the enzymatically catalyzed reaction. As a consequence of the high rates found with cysteine and HCl, all the experiments with tissue homogenates included mixtures with tissue that had been denatured before incubation was started.

The first sea lion tissues to be tested for MeHg-demethylase activity were kidney and liver (male sea lion No. 7). Since these tissues had been stored in the frozen state, it appeared more appropriate to homogenize them by grinding in liquid nitrogen. However, no demethylation occurred when homogenates from these tissues were tested. Mercury analysis and the search for radioactive metabolites gave similar negative results. No radioactive CO_2 was trapped and no radioactivity was recovered by paper chromatography of the incubation mixture supernatant. This meant that no $H^{14}CHO$ had been formed and that the substrate ($^{14}CH_3HgCl$) had not been bound by the added glutathione (a purple ninhydrin-spot with the same R_f-value as for the GSH standard was seen on the chromatograms of the sample supernatants).

The extraction and clean up procedure of Westöö (1968) scaled down for these experiments was checked for recovery of MeHg. To this end, aliquots were taken from both the organic and the aqueous phases after each extraction and counted by liquid scintillation counting. The flowsheet for the extraction procedure is given in Figure 21 where a typical example (whole homogenate of frozen sea lion liver containing 250 ppm of added Hg as MeHg) shows the recovery of radioactivity in each step.

The day after fresh sea lion tissue had been obtained, an experiment was conducted with liver and kidney tissue from sea lion No. 19.



sample	total dpm per level	% transfer from phase to phase	% recovery from previous level
(bk)	41, 949	71. 3	90. 5
(1)	40, 820	70. 5	88. 1
(2)	41, 296	68. 9	89. 1
(bk)	29, 640	83. 3	110. 0
(1)	31, 840	59. 8	122. 0
(2)	33, 747	76. 3	129. 0
(bk)	8, 118	83. 3	74. 7
(1)	7, 113	80. 7	69. 9
(2)	6, 773	80. 7	56. 9
(bk)	4, 830	--	108. 2
(1)	4, 324	--	112. 4
(2)	4, 094	--	111. 5

Figure 21. Flowsheet of extraction of $^{14}CH_3HgCl$ (Westöö, 1968) with results of counting. Three samples are followed through the procedure: a tissue blank (incubation of MeHg with denatured tissue) and duplicate liver samples (whole homogenate of sea lion liver) incubated with 250 ppm methylmercury. Counting of small aliquots (50 µl) was performed after each extraction and after separation of the phases.

The tissues were homogenized in liquid nitrogen and incubated as described above with 125 ppm and 31 ppm mercury as MeHg for 12 h. No demethylation was found by GLC analysis of the recovered MeHg after this period.

To determine whether the microsomal enzyme system was active in these preparations, 4 μmol of aminopyrine were incubated under the same conditions with 200 mg of sea lion liver homogenate. The liver had been stored frozen for one month and the homogenate was prepared with liquid nitrogen. After 30 min incubation time, no formaldehyde could be seen with Nash-reagent, indicating that the mixed-function oxidase system was inactive in these tissue preparations.

The experiment was repeated with liver homogenate from sea lion No. 19 after the liver had been stored for almost three months. This time Mazel's procedure (Mazel, 1971) was exactly followed. The tissue was thawed first, minced, and homogenized in a Potter-Elvehjem-homogenizer at 0-4° C. The supernatant from 250 mg tissue obtained following centrifugation at 10,000 x g for 10 min was incubated with 5 μmol aminopyrine (30 min) and 8 ppm MeHg (16.5 h). This time formaldehyde was detected in the aminopyrine samples and demethylation of MeHg was found to a small, but significant extent. The results of the GLC analysis are shown in Table VII. The figures for the blanks were somewhat higher than for the samples, indicating

more MeHg in the blanks; but it was difficult to decide whether this difference was due to demethylation or variations in the procedure of analysis.

TABLE VII. RESULTS OF GLC ANALYSIS FOR DEMETHYLATION EXPERIMENT WITH SEA LION LIVER SUPERNATANT

Treatment	blank[a]		sample[b, c]			
	DTE	no DTE	DTE		no DTE	
			1	2	1	2
GLC peak height (cm) (analysis on first day)	11.90	10.88	9.55	7.70	11.05	7.75
GLC peak height (cm) (analysis on second day)	12.15	12.75	10.15	8.50	11.10	8.70

[a] 10,000 x g supernatant denatured with $ZnSO_4$-$Ba(OH)_2$ and incubated with 8 ppm Hg as MeHg.

[b] 10,000 x g supernatant as explained in text, incubated with 8 ppm Hg as MeHg.

[c] Samples are 21.9 ± 13.0% lower than blanks and this difference is significant (α=0.01). There is no significant difference (α=0.05) between samples with and without DTE.

Statistical analysis (see APPENDIX IV) showed that there was a significant difference (99% probability) between blanks and samples, denatured and native homogenate. No significant difference (95% probability) was found between samples with and without DTE. The average rate of demethylation was 21.9 ± 13.0%[8] in 16.5 h.

[8] ± standard deviation.

DISCUSSION

Mercury

The similarity between mercury and cadmium has been pointed out. In the present study, the main differences with respect to physiological effects are probably due to the different chemical forms that are encountered by aquatic animals.

Mercury is passed on through the food chain as methylmercury, and therefore tends to accumulate in a more hydrophobic environment represented by the particulate fractions of the cell. Buhler and Mate (1975) have demonstrated *in vivo* demethylation of methylmercuty in sea lion liver. In this thesis (Part III) it could be shown *in vitro* that at least some of the biodegradation takes place in the 10,000 x g x 10 min supernatant of sea lion liver, which includes microsomes and the soluble fraction. Preparations with an inactive mixed function oxidase system did not catalyze demethylation suggesting that the microsomal fraction was the active ingredient. Nothing can be said about the nuclear and mitochondrial fractions where most of the mercury is located. It is not known whether microsomes are the only cell organelles to convert methylmercury to inorganic mercury, and if so, how the methylmercury is transported to its degradation sites.

If the difference in methylmercury content between blanks and

samples in the last experiment described in Part III is indeed due to demethylation, sea lions display unique features, because in vitro demethylation of MeHg has never before been shown to be catalyzed by liver tissue of higher animals. It would be interesting and necessary to reproduce these results, but as wild and protected species sea lions are not easily obtainable, and since they are large aquatic animals, expensive holding facilities are required to keep them as experimental animals. Therefore, the present work was stopped short of the assigned goal of confirming and localizing the demethylating system.

Cadmium

In the case of mercury, marine predators are dealing with a lipophilic substance (MeHg) that may or may not be broken down eventually to yield a more hydrophilic metabolite (Hg^{2+}) with an accompanying redistribution of the element (Norseth, 1972).

With cadmium, the case seems to be more straightforward. The metal enters the organism as Cd^{2+} ion and is distributed according to its properties of binding and solubility. It is found in the soluble fraction of the cell much more so than mercury as the distribution data (Tables III and IV) and the slopes of the regression lines for cadmium in Figure 11 indicate. Cadmium seems to be much more abundant in most marine food chain organisms than mercury. This

may be the explanation for the high cadmium values accumulated in livers and kidneys of the predacious marine mammals. But why then, are cadmium levels so low in the cytosol of sea lion liver cells? Several explanations are at hand: inorganic mercury that is being released slowly or ingested in small amounts may block the cadmium binding sites in this fraction, or inhibit cadmium transport or alter membrane permeability for cadmium; or cadmium may be less abundant in the prey species of sea lions than in the food organisms of arctic marine mammals. Testing the first possibilities would involve difficult laboratory experiments, whereas the last question could be resolved more readily.

Sources of Heavy Metal Contamination in the Studied Animals

Examination of the prey species could as well shed light on the disparity in mercury levels between arctic and temperate species. A similar case has been reported by Gaskin et al. (1973), who found higher mercury concentrations in harbor seals from the Bay of Fundy than from outside the bay. One cannot exclude the possibility that harbor seals prey on different species with different mercury contamination inside and outside the Bay of Fundy. But it seems more likely that the food species are the same, but their level of contamination differs regionally. In this case the mercury concentration in the local environment was reflected in the seals. Enclosed bays would be expected to be subject to pollution to a greater extent

than the open ocean with relatively less shoreline and a greater volume of water for dilution.

The same arguments apply for the difference in mercury concentrations between the sea lions and the arctic marine mammals in the present study. The present case is not as simple, however, because of the difference in feeding habits. There is a marked difference in mercury concentrations, for instance, between bearded seals (mean of 3 livers = 2.7 ppm) and walrus (mean of 5 livers = 0.71 ppm--see Table II) even though both species live in the same habitat. Different feeding habits and corresponding differences in metal levels in their food organisms are the most likely explanation for these differences.

Harbor seals are a better choice to study the amount of pollution in areas far apart, because they tend to remain in a specific area, but are geographically widespread as a species.

Subcellular and Within-Cytosol Distribution of Cadmium and Mercury

As apparent from Figures 8-11, the concentration dependent behavior of heavy metal distribution within the cell and among different soluble proteins follows the same general pattern in liver as well as in kidney. A biexponential relation was found to quite well fit the data expressing the accumulation of heavy metals by a compartment

of high initial affinity (metallothionein or the cytosol) dependent upon accumulation in the corresponding system (the cytosol or the whole cell). These biexponentials describe biphasic curves in a semi-logarithmic plot. Generally, most of the liver data were found on one branch of a specific curve and the kidney values on the other branch, but this was not true without exception (most exceptions occurred with mercury--Figures 8c, 9c, and 10c). Also liver and kidney results can change positions depending on whether cadmium or mercury is plotted. In the case of cadmium distribution, most of the kidney data were scattered about the upper branch of the curve and the liver data about the lower one; and vice versa in the case of mercury. These facts led to the idea that features common to most cells govern the process of heavy metal distribution rather than features that are unique to a given organ. In the light of this idea, it would be interesting to test whether the heavy metal distribution in other marine mammal tissues follows the same general pattern.

If the two phases observed with all curves in Figures 8 to 10 are not due to the differences between livers and kidneys, they might reflect the presence of two types of binding sites with different affinities for heavy metals. Cadmium distribution between the whole cell and the cytosol exhibited a fast initial rate of accumulation in the cytosol followed by a slow rate of accumulation in this fraction. With mercury this second rate was almost zero suggesting the

presence of only one type of binding sites which is saturated in the first phase, so that no more mercury is taken up by the cytosol in the second phase.

This latter pattern was also shown by metals in metallothionein when plotted versus metals in whole tissue or in the supernatant: first a fast rate of accumulation, then a rate near or equal to zero, indicating saturation. Saturation cannot be proven by the small number of analysis data in this study. The graphs in Figure 11 show that an expression that does not have a horizontal asymptote but is increasing slowly and steadily, can be fitted to the data equally well. Hence, many possible explanations exist for heavy metal binding by metallothionein. The two phases can be interpreted as due to two types of binding sites with different affinities, or as one type of binding sites which is saturated in the first phase. Or the first phase may be due to heavy metal accumulation by the metallothionein already present in the cell and the second, slow phase to biosynthesis of metallothionein caused by the presence of heavy metals. Or even both phases may be due to metallothionein biosynthesis for which then two different rates depending on heavy metal concentration have to be postulated. In any case, the finding of a wide range of heavy metal concentration in the cell and the cytosol having little or no influence on the heavy metal concentration in the metallothionein fraction was unexpected.

In principle, many curves could have been used to describe the

data. But exponential functions are quite commonly found to govern natural processes like chemical reactions, radioactive decay, and accumulation and excretion of drugs by organisms, to name just a few.

Heavy Metal Binding to Soluble Proteins in Liver and Kidney

The mercury concentrations in sea lion livers mentioned in this thesis are among the highest ever reported for healthy animals, and cadmium concentrations in the kidneys of the arctic marine mammals are comparably high as well. Thus it was surprising to find no higher amounts of metallothionein (Part II) in their livers and kidneys, than in normal laboratory rats as reported by Piotrowski et al. (1973a). Also the lack of correlation between heavy metal concentration and metallothionein content is disturbing in the light of the theory of induction of metallothionein biosynthesis by heavy metals. Possible explanations to defend the theory are that heavy metal concentrations were still insufficient for induction, or excessive errors in the assay method, which previously had been used with laboratory animals. More groundwork for application of the method to marine mammals has to be laid, and more confidence must be gained by routine assays with rats or other experimental animals.

All gel filtration chromatography shown in Figures 5 and 6 (Part I) and Figure 12 (Part II) indicates high concentrations of heavy

metals in the soluble fractions of marine mammal liver and kidney. Contrary to expectations, metallothionein was not as effective in its role as a "heavy metal buffer" at the high metal concentrations as it was at low concentrations. Therefore, it is tempting to interpret the high concentrations of cadmium randomly distributed in the proteins of the cytosol of bearded seal No. 4 and walrus No. 4 kidneys as evidence for the "onset of toxic effects" as Clarkson (1972) puts it.

Similarly, no single mercury carrier compound could be detected in the high MW fraction of sea lion liver cytosol. Efforts to isolate a high MW mercury binding protein resulted in a "30-fold" purification of certain mercury rich fractions. But much of the initial mercury was discarded in each purification step because of non-specific binding to many proteins, and a considerable percentage of the metal was lost in various treatments, so that only a small fraction of the initial mercury was left to work with. A "30-fold" purification refers to a ratio of ng Hg/mg protein that was increased 30 times. This was the case for a ratio of 200, since the same ratio for the raw 48,000 x g supernatant was about 6.5. A more realistic purification ratio is hard to estimate if the criterion for purity is as unspecific as mercury binding. That is to say, the ratio of mercury/protein does not always increase as the target mercury binding protein of 150,000 daltons is separated from other proteins. Especially separating it from metallothionein will decrease this figure, even

though it is purification in terms of the desired protein.

In the case of metallothionein, a clear-cut interdependence of amount of protein and extent of heavy metal contamination could not be shown, and in liver of sea lion No. 19, the animal with the highest measured mercury concentration, no significant heavy metal binding constituent of high MW could be found. At present, the mechanisms of protection against high concentrations of heavy metals in marine mammals are still elusive. As mentioned in the introduction, the element selenium may be the key to the question.

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APPENDICES

APPENDIX I. ANALYSIS RESULTS FOR SUBCELLULAR FRACTIONS OF INDIVIDUAL MARINE MAMMALS.

	Fraction[a]	N	Mc	Ms	S	Total	N	Mc	Ms	S	Total
	Animal	Sea Lion No. 19[b]					Sea Lion No. 20[b]				
LIVER	Protein (mg/g tissue)	36.6	39.4	0.58	524	600.6	68.8	27.3	0.73	384.0	480.7
	Protein (% per fraction)	6.1	6.6	0.1	87.2	100	14.3	5.7	0.2	79.9	100.1
	Hg (μg/g tissue)	30.4	12.0	6.0	2.2	50.6 ppm	45.77	18.94	3.4	2.07	70.2 ppm
	Hg (% per fraction)	60.0	23.7	11.9	4.4	100	65.2	27.0	4.9	2.9	100
	Cd (μg/g tissue)	0.20	0.32	0.10	0.58	1.20 ppm	0.69	0.07	0.05	1.71	2.52 ppm
	Cd (% per fraction)	16.6	26.7	8.7	48.1	100.1	27.2	2.7	2.0	68.0	99.9
	Total tissue analysis:	125 ppm Hg; 1.46 ppm Cd					65 ppm Hg; 1.78 ppm Cd				
KIDNEY	Protein (mg/g tissue)	40.6	30.6	4.6	296.8	372.6	45.2	16.3	3.65	257.2	322.3
	Protein (% per fraction)	10.9	8.2	1.2	79.7	100	14.0	5.0	1.1	79.8	99.9
	Hg (μg/g tissue)	4.74	1.84	1.62	1.88	10.1 ppm	0.51	0.30	0.06	0.67	1.54 ppm
	Hg (% per fraction)	47.0	18.3	16.1	18.7	100.1	33.3	19.6	3.9	43.1	99.9
	Cd (μg/g tissue)	1.92	2.26	0.52	10.2	14.9 ppm	1.39	1.04	0.15	7.1	9.68 ppm
	Cd (% per fraction)	12.9	15.2	3.5	68.4	100	14.3	10.8	1.5	73.3	99.9
	Total tissue analysis:	12 ppm Hg; 13.1 ppm Cd					1.4 ppm Hg; 8.43 ppm Cd				
	Animal	Bearded Seal No. 1					Bearded Seal No. 2				
LIVER	Protein (mg/g tissue)	59.7	3.3	3.7	93.5	160.1	51.6	1.4	3.2	112.0	168.3
	Protein (% per fraction)	37.3	2.1	2.3	58.4	100.1	30.7	0.8	1.9	66.5	99.9
	Hg (μg/g tissue)	2.75	0.12	0.13	0.82	3.82 ppm	0.20	0.02	0.02	0.13	0.36 ppm
	Hg (% per fraction)	72.1	3.1	3.3	21.5	100	71.5	5.7	5.7	17.1	100
	Cd (μg/g tissue)	0.76	0.08	0.10	2.85	3.80 ppm	0.98	0.11	0.12	3.86	5.06 ppm
	Cd (% per fraction)	20.1	2.2	2.7	75.0	100	19.3	2.2	2.3	76.2	100
	Total tissue analysis:	4.0 ppm Hg; 4.33 ppm Cd					0.74 ppm Hg; 6.24 ppm Cd				
KIDNEY	Protein (mg/g tissue)	88.3	1.0	0.3	64.0	153.6	46.0	10.7	1.6	68.6	117.2
	Protein (% per fraction)	57.4	0.7	0.2	41.6	99.9	39.2	0.9	1.4	58.5	100
	Hg (μg/g tissue)	0.21	0.02	0.01	0.43	0.67 ppm	0.09	0.03	0.01	0.14	0.26 ppm
	Hg (% per fraction)	32.3	2.4	2.0	63.2	99.9	32.8	11.1	4.1	52.0	100
	Cd (μg/g tissue)	6.15	0.38	0.28	16.13	22.6 ppm	6.91	0.37	0.37	22.63	30.28 ppm
	Cd (% per fraction)	26.8	1.6	1.2	70.3	99.9	22.8	1.2	1.2	74.7	99.9
	Total tissue analysis:	0.91 ppm Hg; 23.9 ppm Cd					0.39 ppm Hg; 31.4 ppm Cd				

[a] N = nuclei and cell membranes; Mc = mitochondria; Ms = microsomes; S = soluble (cytosol). The total of the subcellular fractions may be compared with the results of the total tissue analysis given below each set of data.

[b] From Lee et al. (1976).

APPENDIX I. (Continued)

	Fraction[a]	N	Mc	Ms	S	Total	N	Mc	Ms	S	Total
	Animal	Bearded Seal No. 4					Walrus No. 2				
LIVER	Protein (mg/g tissue)	83.1	5.6	4.0	100.2	192.9	62.6	2 8	4.0	95.0	164.4
	Protein (% per fraction)	43.1	2.9	2.1	51.9	100	38.1	1.7	2.4	57.8	100
	Hg (µg/g tissue)	1.46	0.08	0.06	1.23	2.83 ppm	0.37	0.02	0.02	0.52	0.93 ppm
	Hg (% per fraction)	51.7	2.6	2.0	43.6	99.9	39.9	2.2	1.6	56.2	99.9
	Cd (µg/g tissue)	2.97	0.23	0.13	11.3	14.63 ppm	1.70	0.12	0.07	9.7	11.57 ppm
	Cd (% per fraction)	20.3	1.5	0.9	77.3	100	14.8	1.0	0.6	83.5	99.9
	Total tissue analysis:	3.4 ppm Hg; 22.8 ppm Cd					0.6 ppm Hg; 8.0 ppm Cd				
KIDNEY	Protein (mg/g tissue)	173.5	11.6	14.9	81.9	281.9	49.7	2.9	3.2	90.4	146.2
	Protein (% per fraction)	61.5	4.1	5.3	29.1	100	34.0	2.0	2.2	61.8	100
	Hg (µg/g tissue)	0.31	0.03	0.04	0.77	1.15 ppm	0.13	0.01	0.01	0.24	0.39 ppm
	Hg (% per fraction)	26.9	2.9	3.3	66.8	99.9	32.6	3.5	3.0	60.8	99.9
	Cd (µg/g tissue)	12.33	1.26	1.93	36.18	51.67 ppm	10.11	0.57	1.0	13.82	25.5 ppm
	Cd (% per fraction)	23.9	2.4	3.7	70.0	100	39.6	2.2	3.9	54.2	99.9
	Total tissue analysis:	1.59 ppm Hg; 65.5 ppm Cd					0.12 ppm Hg; 14.0 ppm Cd				
	Animal	Walrus No. 4					Walrus No. 5				
LIVER	Protein (mg/g tissue)	28.3	1.8	2.3	96.8	130.5	26.1	0.5	3.6	79.0	109.3
	Protein (% per fraction)	21.7	1.4	1.8	74.2	99.1	23.9	0.5	3.3	75.3	103
	Hg (µg/g tissue)	0.46	0.01	0.03	0.49	1.0 ppm	0.44	0.02	0.12	0.57	1.15 ppm
	Hg (% per fraction)	46.3	1.4	2.6	49.7	100	38.0	1.4	10.9	49.7	100
	Cd (µg/g tissue)	2.97	0.04	0.14	10.34	13.5 ppm	2.39	0.27	0.40	5.80	8.85 ppm
	Cd (% per fraction)	22.0	0.03	1.0	76.6	99.63	27.0	3.0	4.5	65.5	100
	Total tissue analysis:	0.91 ppm Hg; 9.33 ppm Cd					0.91 ppm Hg; 8.41 ppm Cd				
KIDNEY	Protein (mg/g tissue)	8.4	2.0	0.7	67.3	78.4	67.2	1.7	1.3	58.5	128.7
	Protein (% per fraction)	10.7	2.5	0.9	85.8	99.9	52.2	1.3	1.0	45.5	100
	Hg (µg/g tissue	0.11	0.03	0.01	0.07	0.21 ppm	0.15	0.01	0.01	0.18	0.35 ppm
	Hg (% per fraction)	49.5	13.0	6.5	30.9	99.9	43.5	1.6	3.0	51.9	100
	Cd (µg/g tissue	22.5	6.26	2.42	42.8	73.94 ppm	28.03	0.92	0.77	33.77	63.5 ppm
	Cd (% per fraction)	30.4	8.5	3.3	57.9	100.1	44.1	1.5	1.2	53.2	100
	Total tissue analysis:	0.23 ppm Hg; 72.8 ppm Cd					0.15 ppm Hg; 70.1 ppm Cd				

[a] N = nuclei and cell membranes; Mc = mitochondria, Ms = microsomes; S = soluble (cytosol). The total of the subcellular fractions may be compared with the results of the total tissue analysis given below each set of data.

APPENDIX II. RECOVERY OF HEAVY METALS AFTER GEL CHROMATOGRAPHY ON G-75 AND CORRECTIONS.[a]

		µg/g Tissue in the Cytosol	µg/g Tissue from Column Fractions	% Recovery	Correction
LIVER					
Sea Lion					
No. 19	Cd	0.58	0.41	71.2	--
	Hg	1.6	2.11	132	1.16 → 2.2
No. 20	Cd	0.62	1.68	271	0.62 → 1.7
	Hg	2.07	2.21	106.8	--
Walrus					
No. 2	Cd	9.7	4.35	44.9	4.35 → 9.61
	Hg	0.52	0.89	170	--
No. 4	Cd	10.34	5.87	56.8	--
	Hg	0.49	0.28	58	--
No. 5	Cd	5.8	4.1	70	--
	Hg	0.57	0.21	37.5	0.21 → 0.58
Bearded Seal					
No. 1	Cd	2.85	3.24	114	--
	Hg	0.82	0.82	100	--
No. 4	Cd	7.02	11.32	161	7.02 → 11.3
	Hg	1.23	0.83	67.8	--
KIDNEY					
Sea Lion					
No. 19	Cd	7.16	10.16	142	7.16 → 10.2
	Hg	1.88	1.63	86.7	--
No. 20	Cd	4.22	7.07	168	4.22 → 7.1
	Hg	0.59	0.67	114	0.59 → 0.67
Walrus					
No. 2	Cd	13.82	8.80	69.1	--
	Hg	0.24	0.16	67.9	--
No. 4	Cd	42.8	21.6	50.4	21.6 → 42.8
	Hg	0.06	0.04	71.7	--
No. 5	Cd	24.23	33.77	71.7	--
	Hg	0.18	0.09	48.9	0.09 → 0.17
Bearded Seal					
No. 1	Cd	16.13	15.14	93.9	--
	Hg	0.43	0.43	100	--
No. 4	Cd	36.18	11.77	32.5	11.77 → 36.18
	Hg	0.77	0.64	83.1	--

[a] Corrections were made according to results of whole tissue analysis.

APPENDIX III. PARAMETERS FOR THE REGRESSION LINES IN FIGURES 8, 9, 10, and 11.

No. of Figure & designation of axes[a]	Parameters A_1 and A_2	95% Confidence Interval	Parameters B_1 and B_2	95% Confidence Interval	R_1^2 & R_2^2	F_1 & F_2[b]	Points deleted for regression[c]	Identification of deleted points[d]
8a	85.95	(32.46 - 227.6)	0.0003019	(± 0.0006443)	0.2082	1.315	6, 10, 13	W2k, 5L20L, SL191
MT - T	294.9	(219.7 - 395.8)	-0.02399	(± 0.007502)	0.8859	54.37**		
8b	73.84	(36.51 - 149.3)	0.0005673	(± 0.0005840)	0.4680	5.278	5, 9	W5L, W2K
MT - T Cd	110.2	(88.74 - 136.9)	-0.0262	(± 0.005095)	0.9265	100.8**		
8c	3.457	(1.172 - 10.61)	0.0005258	(± 0.001206)	0.2391	1.257	3	W4K
MT - T Hg	3.311	(2.720 - 4.029)	-0.07180	(± 0.01455)	0.9397	124.6**		
9a	57.11	(21.78 - 149.8)	0.001744	(± 0.003555)	0.2238	1.441	--	
MT - S	114.2	(63.22 - 206.3)	-0.03377	(± 0.2713)	0.5196	8.204*		
9b	56.62	(21.73 - 147.6)	0.001746	(± 0.003459)	0.2338	1.526	--	
MT - S Cd	87.89	(48.00 - 160.9)	-0.03273	(± 0.02750)	0.5184	7.534*		
9c	1.977	(1.004 - 3.891)	0.08178	(± 0.06516)	0.7225	10.41*	5, 6, 10	W2L, W5L, W4L
MT - S Hg	2.824	(1.457 - 5,472)	-0.4130	(± 0.3431)	0.6345	8.679*		
10a	81.25	(63.89 - 103.3)	0.002294	(± 0.0005778)	0.9098	80.67**	12, 15	5L20L, SL19L
S - T	2564	(1513 - 4342)	-0.06496	(± 0.04086)	0.7024	14.14*		
10b	66.22	(52.60 - 83.36)	0.002706	(± 0.0006649)	0.8772	78.61**	--	
S - T Cd	149.1	(96.71 - 230.0)	-0.04196	(± 0.02238)	0.6598	17.46**		
10c	6.065	(2.457 - 14.97)	0.001128	(± 0.001433)	0.5059	4.095	--	
S - T Hg	5.819	(3.594 - 9.423)	-0.1232	(± 0.08228)	0.5205	10.85**		
11a Cd	19.69	(15.27 - 25.39)	0.02931	(± 0.008239)	0.8175	58.22**	--	
T - MT Hg	1.041	(0.8312- 1.304)	1.110	(± 0.2702)	0.8565	77.60**	--	
11b Cd	16.02	(11.80 - 21.75)	0.02692	(± 0.009593)	0.7360	36.24**	--	
S - MT Hg	1.196	(0.8631- 1.656)	0.4844	(± 0.1887)	0.6999	30.32**	--	
11c Cd	34.08	(27.14 - 42.81)	0.009354	(± 0.002345)	0.8266	71.51**	--	
	0.9251	(0.7762- 1.103)	0.5372	(± 0.09951)	0.8972	131.0**	--	

[a] The first capital letter designates the ordinate (logarithmic), the second capital letter designates the abscissa (linear). MT stands for nmol of heavy metals per 1 g tissue in the metallothionein fraction. T means nmol of heavy metals per 1 g of whole tissue. S abbreviates nmol of heavy metals per 1 g tissue in the cytosol (soluble fraction). Cd and Hg indicate whether cadmium or mercury is plotted. If the chemical symbol is omitted, the plot consists of nmol Cd plus nmol Hg.

[b] Asterisks indicate the probability for slopes different from zero. *: $P \geq 95\%$; **: $P \geq 99\%$.

[c] The points in each graph are numbered according to increasing value of the abscissa.

[d] Deleted points are identified as to which animal and which organ they belong to. BS = bearded seal, SL = seal lion, W = walrus, K = kidney, L = liver.

APPENDIX IV. STATISTICAL ANALYSIS OF THE DEMETHYLATION EXPERIMENT (TABLE VII).

The data can be described by the following model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon,$$

where y_{ijk} = yield in cm peak height for the i^{th} value of α, the j^{th} value of β, and the k^{th} value of γ.

μ = overall mean of the observations.

α = the added effect of factor 1 (blank or sample).

β = the added effect of factor 2 (DTE or no DTE).

γ = the added effect of the block (1st or 2nd day).

ε = the random error, assumed to be normally distributed with a mean of zero and a variance of σ^2.

α, β, γ, and ε are measured as deviations from μ.

Two hypotheses are to be tested:

1) There is no significant difference between blanks and samples.

H_o: $\alpha = 0$.

2) There is no significant difference between adding or leaving out DTE. H_o: $\beta = 0$.

Analysis of Variance[a]

Source	Degrees of Freedom	Mean Square	F
Total	11		
Factor 1	1	18.1134	11.3692**
Factor 2	1	0.4313	0.2707 ns
Block	1	1.7063	
Error	8	0.5932	

Standard deviation, S. D. = 1.262

[a]Analysis of variance calculated by computer OS-3 using SIPS (statistical interactive programming system).

APPENDIX IV. (Continued)

Contrasts:

		blank		sample	
	n	2	2	4	4
		DTE	no DTE	DTE	no DTE
	$\bar{y}$	12.0250	11.8125	8.9750	9.6500
	μ	μ_1	μ_2	μ_3	μ_4
(blank vs. sample)	L_1	+1	+1	-1	-1
(DTE vs. no DTE)	L_2	-1	+1	-1	+1

(A linear function, of a set of means
$L = k_1\mu_1 + k_2\mu_2 + \ldots + k_p\mu_p$
is a contrast if the k_i are a set of constants such that

$$\sum_{i=1}^{p} k_i = 0$$

provided that at least one $k_i \neq 0$. If L is a contrast, then the sample estimate of L, $\hat{L}$, is
$\hat{L} = k_1\bar{y}_1 + k_2\bar{y}_2 + \ldots + k_p\bar{y}_p$,
where $\bar{y}_i$ is the sample estimate of μ_i.)

$\hat{L}_1 = 12.025 + 11.8125 - 8.975 - 9.65 = 5.2125$

$\hat{L}_2 = 11.8125 - 12.025 + 9.65 - 8.975 = 0.4625$

Sample estimate of the variance, $\hat{V}(\hat{L}_1)$:

$(k_1^2/n_1 + k_2^2/n_2 + k_3^2/n_3 + k_4^2/n_4)MSE = (1/2 + 1/2 + 1/4 + 1/4)\ MSE =$

2.389 (same variance for $\hat{L}_2$)

S. D $= \sqrt{\hat{V}(\hat{L})} = 1.546$

t-values calculated with data	Critical t (table)
L_1: $t = \frac{\hat{L}}{\sqrt{\hat{V}(\hat{L})}} = 3.372$	$t_{0.05\ (8)} = 2.306$
L_2: $t = 0.299$	$t_{0.01\ (8)} = 3.355$

For L_1, t is the critical region, reject H_o. The mean peak heights of MeHg for the samples incubated with sea lion liver

APPENDIX IV. (Continued)

homogenate supernatant are highly significantly (α=0. 01) different from those of MeHg incubated with denatured supernatant. For L_2, t is not in the critical region. Fail to reject H_o. There is no significant (α=0. 05) difference between samples with and without DTE.