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A naturally occurring cadmium and zinc binding protein from the liver and kidney of *Fulmarus glacialis*, a pelagic North Atlantic seabird

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Since the first description of metallothionein [1], many similar metal binding proteins have been described in a variety of species [2–6] and it has been suggested that the induction of metallothionein synthesis, following administration of certain heavy metals, is a way in which the toxic effects of such metals as cadmium and mercury can be limited [2, 7]. The analyses of some puffins (*Fratercula arctica*) and other seabirds from around the British coast [8, 9] indicated that some species carry high loads of cadmium in comparison with the levels found in other marine organisms from British waters [10]. Pacific and Antarctic seabirds have been found with high mercury and cadmium residues [11], and recent data obtained from birds collected on Saint Kilda, U.K., showed that North Atlantic seabirds contained some of the highest levels of cadmium yet recorded for a living wild animal, up to 240 mg kg^{-1} dry weight, in the kidney [12]. The highest levels of cadmium were found in two species of petrel, the Manx shearwater (*Puffinus puffinus*), and the northern fulmar (*Fulmarus glacialis*), which also contained the highest mercury concentrations, up to 46.4 mg kg^{-1} dry weight, in the liver [13].

Both mercury and cadmium are known to have deleterious effects on reproductive physiology, and, in order to assess the significance of the metal concentrations found in seabirds, an investigation into the role of methalothionein has been conducted, since, if much of the metal in these animals is bound to this protein, they may be able to tolerate apparently toxic levels of heavy metals.

The presence of the high metal residues in a wild population also afforded a rare opportunity to examine metal-metallothionein relationships which have developed over a long period in a natural, rather than in a laboratory situation.

MATERIALS AND METHODS

Birds were netted on the cliffs of Saint Kilda, U.K., exsanguinated, and their tissues removed and frozen on solid CO_2. Subsequently, tissues were stored at $-20°$ or below until, and after; frozen aliquots were removed.

Tissues were placed in approximately 5 volumes (w/v) of 0.25 M sucrose containing 10 mM Tris buffer pH 7.4, and homogenised with a polytron. A supernatant fraction was obtained by centrifugation of the homogenate at 35,000 *g* maximum for 2 hr at 4°. The supernatant was decanted and 4 ml loaded on to an 86 × 1.6 cm column of Sephadex G-75. Fractions of 3 or 6 ml were collected at a flow rate of 12 ml hr^{-1} with 10 mM Tris buffer pH 7.4 containing 3.1 mM sodium azide as an antibacterial agent. The E_{250} of each fraction was determined since metallothionein characteristically absorbs light at 250 nm. Protein was measured by the Lowry technique [14]. After the addition of 2 ml of nitric acid to each fraction, the metal concentrations were determined by atomic absorption spectroscopy. The limit of detection for each metal was (mg kg^{-1}): Zn 0.03, Cd 0.01, and Hg 0.01. The recovery of all metals was >95 per cent, and the precision of the measurement of the metal content of each fraction was <5 per cent. The method of Alexander [15], was used to detect sulphydryl groups. The decrease in E_{300} of *N*-ethylmalemide (NEM) was determined 20 min after the addition of 1 mM NEM to the fractions. NEM was used in preference to reagents such as *p*-mercuribenzoate, as the presence of a mercurial would have made the subsequent measurement of endogenous mercury extremely difficult.

RESULTS

In each case the 35,000 *g* supernatant contained approximately 80 per cent of the total cadmium content of the tissues. Figure 1 shows the result of the filtration of a kidney aliquot from the fulmar containing the highest level of cadmium, 240 mg kg^{-1} dry weight, found in this organ. The main cadmium peak coincides with a zinc peak, and with a rise in E_{250}. The position of these three coincident peaks suggests that the metals are bound to a protein with a mol. wt of about 10,000, which is probably a metallothionein. The kidney contained too little mercury to make chemical analysis possible, and so the liver of the same animal, which contained 49.4 mg kg^{-1} cadmium and 46.4 mg kg^{-1} mercury (dry weight values), was fractioned and 6 ml fractions were collected. Figure 2 shows that, while both cadmium and zinc were bound to the metallothionein peak, only a small percentage of the mercury was present in these fractions. A marked decrease in the

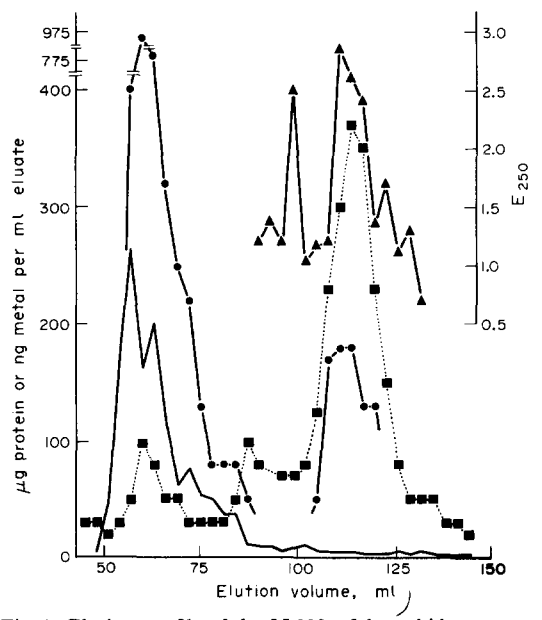

Fig. 1. Elution profile of the 35,000 *g* fulmar kidney supernatant run on an 86 × 1.6 cm column of Sephadex G-75. (——) protein, (●——●) Zn, (■····■) Cd and (▲——▲) E_{250}, which has only been plotted in the region of the metallothionein-like protein.

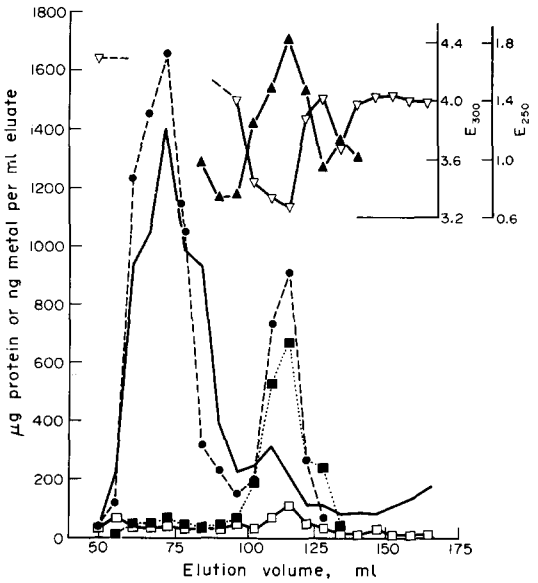

Fig. 2. Elution profile of the 35,000 *g* fulmar liver supernatant run on an 86 × 1.6 cm column of Sephadex G-75. This column was different from that used for the results shown in Fig. 1. Note that although the tissue levels of mercury and cadmium were almost the same, proportionately much less mercury than cadmium appears to be bound to metallothionein. (——) protein, (●——●) Zn, (■· · · ·■) Cd, (□——□) Hg, (▲——▲) E_{250}, which has only been plotted in the region of the metallothionein-like protein, and (▽——▽) E_{300}. The symbol shown ▽-- --▽ indicate a region in which turbidity prevented accurate estimation of E_{300}.

absorbance of NEM, and a rise in E_{250} were seen in the fractions indicated (Fig. 2), and, even though NEM may not react with all the −SH groups in the fractions, the depression of E_{300} indicates that this metal binding protein, like metallothionein, is rich in −SH groups.

DISCUSSION

The results suggest that a protein, similar in many respects to metallothionein, is present in seabirds which have been exposed to cadmium, a natural part of their environment [12]. However, the seabird metallothionein may differ from that which can be induced in rats and rabbits by experimental dosing with heavy metals [2,5] in that it does not appear to bind substantial amounts of the mercury in the tissue. The mercury in seabird tissues may be predominantly in the methyl form [16], and this may explain why mercury did not appear to be bound to the seabird metallothionein, although there is some argument concerning the binding of methylmercury to metallothionein [5, 17].

As none of the seabirds collected in the recent Atlantic study appeared to be suffering from toxic effects of the metals found in its tissues [12] it is possible to conclude that the binding of cadmium to metallothionein probably "detoxifies" the metal. However, it is important to consider the possibility that the normal function of metallothionein is to store zinc, and, in wild animals needing unusual quantities of this metal for such purposes as moult and egg-laying, seasonal variations in metallothionein levels might be expected. There is little evidence to support the idea that this protein can act as a zinc store, but two observations that are consistent with such a view must be noted, (i) the hepatic protein binds more zinc than cadmium, an effect that has been observed before [5], and (ii) zinc appears to be able to induce metallothionein synthesis [7].

If metallothionein is a zinc store and if there are seasonal variations in metallothionein levels, two current ideas about the significance of cadmium in animal tissues must be re-assessed. First, the binding of cadmium to metallothionein may not represent a detoxification of cadmium but a toxic interference with normal zinc metabolism. Secondly, the idea that animals containing high cadmium residues have been feeding on a polluted diet may be incorrect since, if metallothionein levels vary with changes in the animal's physiological state, then at certain seasons more of the normal amounts of dietary cadmium could be retained in the animal than at others.

Thus, although it appears that seabirds with high cadmium residues are healthy because the metal is bound to a metallothionein-like protein, judgements about the toxic significance of the residues must be reserved at least until work currently in progress shows whether there are seasonal variations in metallothionein.

Since only a small fraction of the mercury in the tissue appears to be bound to metallothionein, further work will identify the cell fractions in which the mercury is located, and determine whether it is in the methyl form. Only then can the toxic significance of the high mercury residues found in certain North Atlantic seabirds begin to be assessed.

Evidence is presented which suggests that there is a cadmium and zinc binding protein in the kidney and liver of a pelagic North Atlantic seabird, the northern fulmar (*Fulmarus glacialis*). This protein has many of the properties of metallothionein, although it does not bind the mercury in the tissue. It is suggested that the toxic effects of cadmium may be limited by this protein, but that the possible role of metallothionein as a zinc store must be clarified before firm conclusions can be drawn.

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Institute of Terrestrial Ecology,
Monks Wood Experimental Station,
Abbots Ripton, Huntingdon,
PE17 2LS, U.K.

DANIEL OSBORN

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Maleimide II—interaction with L-asparaginase from *Escherichia coli*

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In functional terms, maleimide, like *N*-ethyl maleimide, is a reagent well known for its ability to react covalently with sulfhydryl functions; on structural grounds this compound also bears a strong resemblance to the hypothetical cyclic anhydride of L-asparagine [1–3]. As a consequence of this resemblance, it might be postulated that maleimide could serve as a substrate for the amido hydrolytic action of L-asparaginase (EC 3.5.1.1). By similar reasoning, maleimide might be expected to compete with L-asparagine as well as with other alternate substrates for the active site on the enzyme. To test these hypotheses, we have examined the interaction of maleimide with crystalline L-asparaginase from *Escherichia coli*.

L-Asparaginase from *E. coli* (EC 3.5.1.1; sp. act. 300 I.U./mg of protein) was a gift to the National Cancer Institute from Merck, Sharpe & Dohme, Rahway, NJ, U.S.A. L-Asparaginase inactivated with L-DONV (>90 per cent inactivated) was kindly donated by Dr. Robert L. Handschumacher of the Yale University School of Medicine, New Haven, CT, U.S.A. L-Glutamate-oxaloacetate transaminase (GOT) (EC 2.5.1.1; sp. act. 180 I.U./mg of protein) was purchased from Boehringer-Mannheim, New York, NY, U.S.A. Maleimide, *N*-ethyl maleimide (NEM), maleamic acid, maleic acid, succinamic acid and succinimide were the products of Aldrich Chemical, Cedar Knolls, NH, U.S.A. β-Cyano-L-alanine and α-ketoglutaric acid were procured from CalBiochem, Gaithersburg, MD, U.S.A. L-[U-^{14}C]asparagine (sp. act. 151 μCi/μmole) was the product of Amersham Searle Corp., Silver Spring, MD, U.S.A. Purity was assessed as previously described [4]. Eppendorf 1500-μl plastic centrifuge vessels, procured from the Brinkman Instrument Co., Silver Spring, MD, U.S.A., were used throughout.

Electrophoretic separation of maleimide, maleamic acid and maleic acid was carried out for 1 hr at 3000 V on Whatman 3MM paper (96 cm) saturated with 0.1 M potassium phosphate buffer, pH 7.0. The spots were identified under ultraviolet (u.v.) light and excised, and their content of the amide or imide was measured by u.v. spectrometry. Under these electrophoretic conditions, maleimide remained at the origin, whereas maleamic acid and maleic acid migrated 3.1 and 5.2 cm respectively.

The hydrolysis of β-cyano-L-alanine and L-asparagine by L-asparaginase from *E. coli*, in the presence and absence of maleimide, was quantified by a coupled spectrophotometric technique for the measurement of L-aspartic acid [5]. Hydrolysis of the other substrates was quantitated with an enzymatic assay for ammonia [6]. In no case was sufficient maleimide sampled to interfere with the stoichiometrics of the ammonia analysis. In cases where the concentration of maleimide in the cuvette would be likely to interfere with the spectrophotometric analysis, an equimolar quantity of mercaptoethanol was added to the incubation mixture prior to sampling for the spectrophotometric assay.

When necessary, a radiometric technique was used to quantify the hydrolysis of L-asparagine, as follows: 5 μl of a freshly prepared maleimide solution of the requisite molarity in 0.1 M Tris–HCl buffer, pH 8.4, or 5 μl of 0.1 M Tris–HCl buffer, pH 8.4, and 5 μl of L-asparaginase solution were driven onto a 5-μl droplet (0.25 μCi; 0.0016 μmole) of L-[U-^{14}C]asparagine contained in the bottom of an Eppendorf 1500-μl centrifuge vessel by a 5-sec acceleration to 12,000 *g*. After a 30-min incubation at 25°, 10 μl of 1 M HCl was added to each vessel and the mixture heated to 95° for 5 min. Then 10 μl of 1 M NaOH was added to each vessel followed by 20 μl of a decarboxylation mixture consisting of 6.8 mM α-ketoglutaric acid, 8.5 mM $ZnSO_4$ in 0.66 M sodium acetate buffer, pH 5.0, containing 20 I.U. GOT/ml. The vessels were incubated for 60 min at 50°. [^{14}C]O_2 arising from the β-decarboxylation of [U-^{14}C]oxaloacetic acid produced by the GOT-catalyzed transamination with α-ketoglutaric acid of any L-[U-^{14}C]aspartic acid liberated was trapped in a 5-μl droplet of 40% (w/v) KOH deposited on the lid of the closed vessels, then counted as previously described [7].

With L-asparagine as substrate, maleimide inhibited L-asparaginase from *E. coli* in a noncompetitive manner* (K_i = 0.05 M) (Fig. 1B), but inhibition was apparent only at relatively high concentrations of inhibitor (0.025 to 0.20 M); no inhibition whatsoever could be detected with

* Inhibition of L-asparaginase by maleimide can possibly be explained by the known proclivity of this agent to react covalently with α-amino acids [8]. Some support for the notion that maleimide can react with L-asparagine was provided by experiments in which the two compounds were incubated together each at a concentration of 0.05 M for 48 hr in sodium phosphate buffer, pH 7.0. Ascending chromatography of the reaction mixture in the systems described by Smyth *et al.* [8] revealed the formation of a new entity yielding a yellow chromogen with ninhydrin and with an R_f of 0.1. However, determination of the rate of disappearance of L-asparagine at 0, 24 and 48 hr in the incubation mixtures of the composition given above revealed a diminution of, at most, 5 per cent. Moreover, this decrement was not progressive with increasing time of incubation. The quantitatively minor extent of the reaction suggests that the covalent interaction of maleimide with L-asparagine is not causative of the inhibitory action of maleimide vs L-asparaginase from *E. coli*.