An Easily Biotinylated, High Molecular Weight Component of Bovine Skim Milk, Which Binds the Radionuclide ^{207}Bi

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The purpose of this investigation has been to identify commonplace, inexpensive substances that might be used to link radioisotopes to antibodies. It was found that a high molecular weight component of bovine skim milk binds bismuth ion and might be used as a carrier of short-lived bismuth isotopes in radioimmunotherapy applications. The bismuth-binding material may be complexed with ^{207}Bi (as the Bi^{3+} cation) and biotinylated using the reagent N-hydroxysuccinimido-D-biotin. Avidin on agarose columns sequestered 69% of the applied biotinylated product (S.D. 12%), whereas only 19% of the bismuth activity adhered to avidin columns that had been pre-blocked with D-biotin (S.D. 7%). Complexed with the short-lived alpha emitter ^{212}Bi, the biotinylated product might be used in pre-targeting modes of radioimmunotherapy without modification. The bismuth-binding agent could be casein micelles, with Bi^{3+} exchanging into Ca^{2+} sites, but this has not been proven. ***©1999 Oklahoma Academy of Science***

INTRODUCTION

The α-emitting isotope, ^{212}Bi (*1*), has received attention as a toxic agent for an antibody-mediated, selective tumor destruction application known as radioimmunotherapy (*2*), where a short-lived radionuclide is linked by some means to an antibody that is selective for a tumor alloantigen. Malignant cells are killed by ionizing radiation (radiolysis) when the antibody binds selectively to their surfaces. Owing to its very short half-life (60.6 min), the applicability of ^{212}Bi and similar isotopes is limited to use against circulating malignant cells such as leukemias and some lymphomas (*3*). Molecular crosslinking strategies have to focus on prepared bismuth carriers with appropriate binding sites because the isotope has to be added immediately before use.

Bismuth carriers tried to date have included antibody-linked, low molecular weight organic chelates such as the isobutylcarboxycarbonic anhydride of diethylenetriaminepentaacetic acid. The latter is selective for Bi^{3+} (*4,5*). Natural metal-binding proteins, such as metallothionines, might also be considered because metallothionine-linked immunoglobulins have been used to transport ^{99m}Tc (*6*), and the Bi(III) cation binds to small, metallothionine-like proteins found in hepatic tissue (*7*). These approaches involve either rare or expensive materials.

We have considered the possibility that a cheap, commonplace bismuth carrier might exist, and after examining a variety of plant and animal materials, a familiar component of milk, the skim milk fraction (which contains casein micelles) has emerged as a potentially appropriate, easily conjugated bismuth carrier. In particular, the casein micelles of milk (*8,9*) *should* bind to bismuth ions for rational reasons. The micelles consist of aggregates of a subunit phosphoprotein held together ionically by Ca^{2+} ions in $Ca_9(PO_4)_6$ clusters, and one may thus envision an exchange of bismuth ions into calcium sites. A strong interaction is expected because the orthophosphate of bismuth is more insoluble than the orthophosphate of calcium. The solubility of $BiPO_4$ is $\sim 10^{-9}$ g L^{-1}, whereas for $Ca_3(PO_4)_2$ it is $\sim 10^{-2}$ g L^{-1} (*10*). The lysine content of casein also suggests the feasibility of crosslinking methods based on N-hydroxysuccinimido functional groups or similar structures that react with primary amines, forming covalent amide bonds. These expectations are supported by the experimental results reported here, but direct proof that casein micelles are the actual binding agents is lacking at this point.

MATERIALS and METHODS

Materials: The bismuth tracer used in this work, ^{207}Bi, was obtained as the nitrate (in dilute nitric acid) from the proton irradiation facility at Los Alamos National Laboratory (Los Alamos NM). Although ^{207}Bi could not be used for the envisioned tumor therapy application, it is much more appropriate for measurements of binding interactions because of its 32.2 year half-life. The sodium borate buffer components (boric acid and sodium tetraborate) and D-glucose were obtained from Aldrich Chemical Company (Milwaukee WI). Materials obtained from Sigma Chemical Company (St. Louis MO) were D-biotin (Sigma B-4501), N-hydroxysuccinimido-D-biotin (abbreviated hereafter as NHS-biotin, Sigma H-1759), Sephadex G-100 gel permeation media (Sigma G-100-120) and avidin on agarose affinity beads (Sigma A-9207). Fresh bovine skim milk was purchased locally and bore the label "Farm Fresh" or "Superbrand".

Chromatographic Procedures: The casein micelle fraction was isolated from other milk components at 25 °C on Sephadex G-100 columns (1 cm x 8 cm). Gels were prepared by swelling 0.5 g of the dry material in 25 mL of a 0.10 mol L^{-1}, pH 8.5, sodium borate buffer and by sedimenting the columns from wet slurries. Columns were calibrated using high molecular weight Blue Dextran. The void fraction containing the pigmented dextran was also turbid and easily visualized.

Affinity chromatography procedures made use of 0.5 mL columns of avidin on agarose beads, which were initially suspended in 0.5 mol L^{-1} NaCl and sedimented over a glass wool plug in 0.5 cm diameter Pasteur pipets (these were fitted with small stopcocks). Elutions were carried out at 25 °C with the 0.10 mol L^{-1} , pH 8.5 sodium borate buffer (the bead column was washed beforehand with this medium).

Equilibration of ^{207}Bi into Milk Protein: In a typical experiment, 100 microliters of the acidic ^{207}Bi stock solution with a measured rate of 223,000 counts per min (cpm) (background = 2,880 cpm) was delivered to the bottom of a small test tube. To this was added 1.00 mL of 10% aqueous glucose, with stirring for 5 min in an ice bath (0 °C). Using 9.0 mol L^{-1} NaOH (or 0.1 mol L^{-1} HCl in the case of an overshoot), the solution pH was adjusted to 8.5 by adding microliter amounts of the NaOH or HCl solutions with continuous stirring, and upon reaching the desired pH, 0.5 mL of skim milk was added. Stirring was continued for an additional 5 min at 0 °C, then stopped. Equilibrating mixtures prepared this way were then incubated without stirring for ≥ 30 min at 0 °C.

The described order of addition is critical, and omission of the 10% glucose solution will result in having very little of the radiolabel in the Sephadex G-100 void fraction. The glucose solution apparently acts to solubilize the bismuth ion and facilitate its transfer to the binding agent in skim milk. The procedure is somewhat wasteful of the radioisotope because only small portions of the purified biotinylation product (10^3 cpm) were applied to avidin affinity columns to avoid saturation of the binding sites. The equilibrations could be scaled down, but the amounts described here were chosen for ease of manipulation.

Further Treatment of Bismuth Labeled Micelles, Including Biotinylation: After completing the above radiolabeling steps, the bismuth-containing (high molecular weight) fraction was isolated from low molecular weight materials by passing the mixture through a fresh Sephadex G-100 column prepared as described above at 25 °C (room temperature), and only the Blue Dextran calibrated void fraction (total volume 3.0 mL) was collected for further procedures. As noted above, this fraction coincides with easily visible turbidity, and the calibration step with Blue Dextran is not essential in practice. The column eluent was 0.10 mol L^{-1} aqueous borate buffer, pH 8.5.

The latter fraction was biotinylated using NHS-biotin. Biotinylation was carried out directly on 2.0 mL of the void (micelle) fraction, still in the 0.10 mol L^{-1} M sodium borate eluting buffer, to which 6 mg of NHS biotin in 300 µL of dimethyl sulfoxide was added with immediate stirring (to mix thoroughly), and further gentle rocking was continued for 10 min. Temperature was maintained at 0 °C in an ice bath during the biotinylation reaction (~6 h).

The product was again passed through a fresh Sephadex G-100 column at 25 °C to remove low molecular weight D-biotin. As before, only the void fraction was kept for further affinity chromatography experiments and activity quantitation.

Instrumentation: The ^{207}Bi isotope used here decays predominantly by K-electron capture, and one has a choice of detecting X-rays emitted by

the daughter nuclide (^{207m}Pb) with a K series between 73 and 88 keV, or the associated gamma radiations at 0.57, 1.06, and 1.77 MeV, the 0.57 MeV emission being preferred because of its higher intensity. However, we chose to measure the X-radiation from the K-capture daughter nuclide by using a Bicron Model 2MW2/2 NaI-type scintillation detector and an Oxford PCA-P 1024 channel energy dispersive gamma spectrometer. The Oxford PCA-P software permits quantitation of regions of interest (i.e., by integration), and only the channels recording the X-ray emissions were counted. Although the X-ray channels have a higher background rate than the gamma channels, the former also has a compensating higher count rate. By taking sufficiently long counts, counting errors were minimized (*e.g.,* 3-h counts, error <1.5 %).

RESULTS and DISCUSSION

A component of ^{207}Bi-treated skim milk elutes in the Blue Dextran (void) fraction of Sephadex G-100 columns. If the isotope is equilibrated with the milk specimen for 30 min at 0 °C, the amount appearing in the void fraction of the initial Sephadex purification (before biotinylation) is about 30% of the total activity present. Equilibrations involving smaller quantities of added isotope resulted in relatively more radiolabel in the low molecular weight fraction, suggesting the presence of a saturable, low molecular weight component with strong affinity for bismuth in milk. We have not attempted to isolate low molecular weight, bismuth-binding substances, and the work to date has been focused on the casein-containing fraction.

The essential nature of the high molecular weight component of skim milk was established in separate control experiments. If the neutralized solution of bismuth ion in glucose is applied directly to the Sephadex column without first adding the milk, most of the label remains on the column and elutes very slowly (apparently due to bismuth(III) ion interacting directly with the Sephadex G-100 column). Less than 2% of the bismuth activity appears in the void volume in this case, and the isotope continues to elute indefinitely (i.e., smearing well beyond the volume where non-interacting low molecular weight materials normally elute). Combined with milk, about 30% of the label elutes with the void fraction, as noted above.

Based on trials with avidin affinity columns, attempts to biotinylate the high molecular weight, bismuth-containing fraction from Sephadex G-100 columns were successful. After biotinylation of the first ^{207}Bi-containing void fraction with NHS-biotin reagent and removal of low molecular weight biotin products on a second (fresh) Sephadex G-100 column, a small aliquot of the putative biotinylated casein component (i.e., from the void fraction) was divided into two equal portions, one of which was passed through an untreated 0.5 mL column of avidin on agarose and the other through an identical avidin on agarose column that had been preblocked with a 600 µL portion of a solution of 10 mg of D-biotin in 6.0 mL of 0.10 mol L^{-1}, pH 8.5, borate buffer. The blocked affinity column had been washed with 15 mL of the borate buffer before the biotinylated material was added. The applied aliquots were small enough to avoid overloading the affinity columns, and each contained ~10^3 cpm of ^{207}Bi. Biotinylation control experiments in which D-biotin was added in place of NHS-biotin resulted in no binding of the radiolabel to the control or complete affinity columns, showing that a covalent crosslink is necessary to achieve the effects reported here.

Quantitation of ^{207}Bi cpm in five independent biotinylations of freshly prepared and radiolabeled skim milk fraction led to the mean values and standard deviations shown in Table 1, which shows that significantly more activity was retained on the untreated avidin columns. The relative affinity of biotinylated material toward the two columns may be visualized because the effluent from the blocked column is more turbid than the effluent from the untreated column (Fig. 1).

These results demonstrate the possibility of using the bismuth binding, high molecular weight component of skim milk as a crosslinkable transport agent for bismuth isotopes. In a practical application involving immunoglobulins, an antibody conjugate with the binding agent would have to be prepared first, then loaded with ^{212}Bi immediately before use. In actual use the diffusion of $^{212}Bi^{3+}$ into a micellar matrix would not involve ponderable amounts of the ion as a consequence of the very high specific activity attainable by the isotope generator method (*1,2*). At such a high specific activity only a fraction of the calcium ions would need to be replaced with $^{212}Bi^{3+}$ to attain thera-

TABLE 1. Results of biotin labeling attempts: Fractions of biotinylated bovine milk-associated ^{207}Bi activity binding to and eluting from affinity columns of avidin on agarose.[a]

	COMPLETE EXPERIMENT		CONTROL EXPERIMENT[b]	
Trial	Column	Effluent	Column	Effluent
1	0.72	0.28	0.11	0.89
2	0.88	0.12	0.13	0.87
3	0.60	0.40	0.27	0.73
4	0.60	0.40	0.20	0.80
5	0.64	0.36	0.23	0.77
mean[c]	0.69	0.31	0.19	0.81
S.D.	0.12	0.12	0.07	0.07

[a] A total of 10^3 cpm of ^{207}Bi (X-ray emission minus background) was applied to each column; three hour counts resulted in a relative error of <1.5% for these measurements, which is significantly less than the observed variations between experiments.
[b] Control columns were pre-blocked with biotin carboxylic acid, then washed with the pH 8.5 borate buffer.
[c] From the data the standard error of the difference between mean values of measured fractions of radioisotope binding to (respectively) complete and control columns is 0.062; The difference between the mean values (0.69 - 0.19) is 0.50, or more than eight standard errors. Thus, the observed differences are highly significant; the probability that the differences are due to a statistical fluctuation is $< 10^{-12}$.

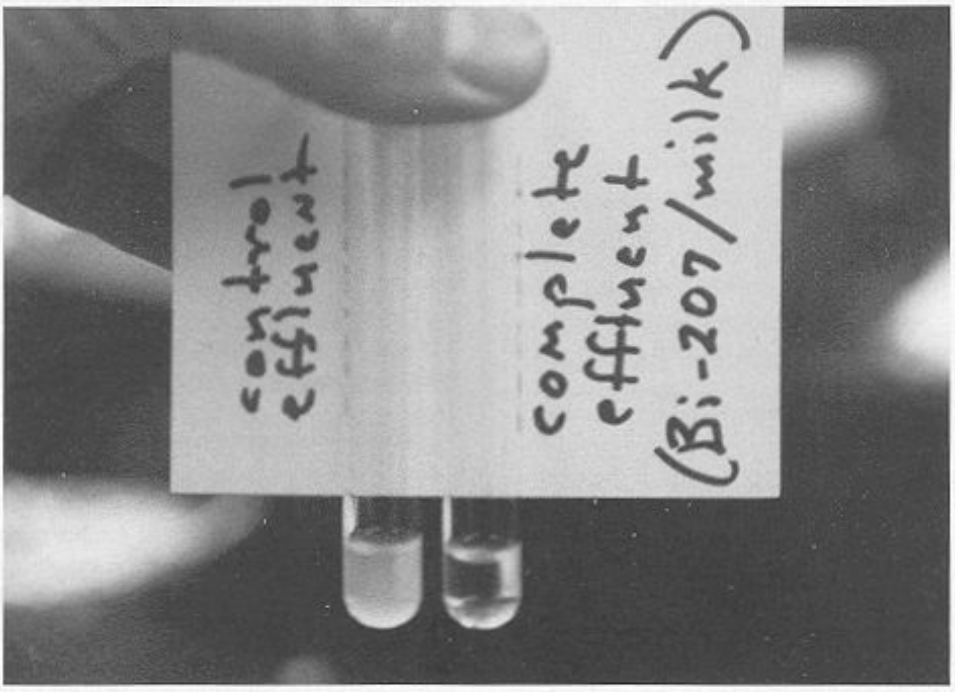

Figure 1. The effluent from D-biotin blocked columns of avidin on agarose (left) is more turbid and contains proportionately more ^{207}Bi activity. The effluent from a column which had not been treated with D-biotin beforehand (right) is less turbid and has a corresponding low level of activity. Both columns were otherwise of identical size and received the same amount of the bismuth-containing, biotinylated component of bovine skim milk (10^3 cpm each).

peutic dose levels. A lethal dose level for suspended cells is in the range 100-200 kBq/mL (*11,12*).

Based on the known properties of casein and its presence in the materials under investigation, we concluded that the binding agent reported here could be micellar casein, but its identity is not proven. The feasibility of crosslinking bismuth to an immunoglobulin by using the milk component also remains to be demonstrated, and an attempt is underway. A direct link might not be necessary due to a new technique described as "pretargeting" (*13*), where an antibody carrying an appropriate (added) binding site is first injected and attaches to the alloantigen on tumor cell surfaces through its F_{ab} receptors. If the binding site carried by the antibody is avidin, for example, the biotinylated bismuth species described here might be used without modification.

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