

Short Communications

A Method for Studying the
Liberation of a Radioprotective
Agent (AET) Bound to Proteins
as a Mixed Disulfide

MAGDOLNA HORVÁTH* and
BENGT MANNERVIK

*Department of Biochemistry,
University of Stockholm,
Box 6409, S-113 82 Stockholm, Sweden*

It has been found that many proteins are able to bind low molecular weight thiols to sulfhydryl groups.^{1,2} The possible importance of such mixed disulfide formation for modulation of enzyme activity, radioprotection, *etc.* is presently unclear.³ The study of these presumptive functions require adequate methods for determination and release of the low molecular weight thiols. In the present communication we describe a method, based on the use of ion exchangers in small columns, for studying the release of the radioprotective agent *S*-2-aminoethylisothiuronium bromide (AET) bound (as an isomer 2-mercaptoethylguanidine, MEG) *via* a disulfide linkage to proteins.

Materials and methods. The mixed disulfide, which is formed between serum albumin and AET (in the form of MEG), was used as a model compound.⁴ Bovine albumin (10 μ mol) was dissolved in 18 ml of 0.2 M phosphate buffer pH 7.5, 35 μ mol of [¹⁴C]-AET in 2 ml of the same buffer were added, and the mixture incubated at room temperature for 24 h. Unbound AET was removed by dialysis against several changes of 20 to 25 volumes of 10 mM phosphate buffer of a pH required in subsequent experiments.

* Present address: "Frédéric Joliot-Curie" National Research Institute for Radiobiology and Radiohygiene, Budapest 22, Hungary.

The protein content of the incubation mixture was determined spectrophotometrically at 278 nm or by the Lowry method.⁵ Radioactivity was measured in a Beckman LS-100 liquid scintillation counter. Generally a ratio of about 0.7 mol AET/mol albumin was found.

The mixed disulfide compounds of equine hemoglobin and bovine insulin were prepared in a similar manner. For details see Horváth *et al.*⁴ The AET content was 2–3 mol/mol Hb or insulin.

Incubations for studying the release of radioactivity upon treatment with thiols were carried out at 30° in 200 μ l incubation mixtures containing buffer, labeled protein, and thiol. After incubation, a 100 μ l aliquot was transferred to an ion exchange column (5 \times 40 mm), which was packed in a Pasteur pipette, and washed into the bed with 0.5 ml of 10 mM sodium phosphate pH 6.5. In pilot experiments 1 ml fractions were collected during the subsequent elution, but in routine experiments a single 5 ml fraction was taken. The radioactivity in the effluent was measured after mixing 1 ml of effluent with 10 ml of toluene-Triton X-100 (2:1) scintillation fluid.⁶

Results. It was assumed that a suitable separation between the negatively charged albumin and liberated MEG, which is positively charged, could be made on DEAE-cellulose columns. Accordingly, it should be possible to measure the release of radioactive MEG from labeled albumin simply by determination of radioactivity in the effluent from a DEAE-cellulose column. It was found that the principle of the measurement worked, but a complication arose due to slow release of radioactivity from the albumin bound to the ion-exchanger. The adsorbed protein was retained as shown by lack of absorbance at 280 nm in the effluent, and, as it had previously been demonstrated that MEG is bound covalently by means of a disulfide bond to albumin,⁴ we conclude that the radioactive group is split off due to microenvironmental effects in the ion-

exchange matrix. The nature of this reaction was not established, but it was found that the sulfhydryl reagents *N*-ethylmaleimide and HgCl_2 did not prevent the decomposition. However, only a minor portion of the total radioactivity was released in the first 5 ml of effluent, which contained the radioactive MEG liberated from labeled albumin by treatment with thiols. This portion could be accounted for in the measurement by control experiments, and the accuracy was therefore sufficient to allow quantitative determinations of the liberation of MEG.

To demonstrate the utility of the technique the reaction of thiols and albumin labeled with $[^{14}\text{C}]$ -AET was studied. Treatment of $0.075 \mu\text{mol}$ labeled albumin with $0.2 \mu\text{mol}$ of unlabeled AET (which isomerizes to MEG at pH-values > 7) at pH 7.5, liberated 75 % of the radioactivity within the shortest possible time of measurement. To investigate the possibility to follow the kinetics, the splitting reaction was run at lower pH values. In these experiments penicillamine, mercaptoethylamine, or glutathione were used. Fig. 1

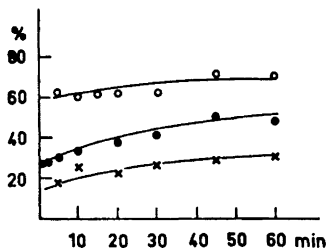


Fig. 1. Release of radioactivity (per cent of total activity) from $[^{14}\text{C}]$ -AET-labeled albumin by treatment with mercaptoethylamine or glutathione at pH 5 for different periods of time. ○, $0.063 \mu\text{mol}$ albumin and $0.2 \mu\text{mol}$ GSH; ●, $0.063 \mu\text{mol}$ albumin and $0.2 \mu\text{mol}$ MEA; ×, $0.063 \mu\text{mol}$ albumin and $0.066 \mu\text{mol}$ MEA.

demonstrates that even at pH 5 a substantial amount of radioactivity is released almost instantaneously, and that glutathione is about twice as active as mercaptoethylamine. It should also be noted that in no case more than 85 % of the radioactivity was released from albumin. Whether this means that part of the label is inaccessible to the reagents used or that

the label is bound covalently not exclusively by disulfide linkage has not been established.

Similar experiments have been carried out with $[^{14}\text{C}]$ -labeled insulin and hemoglobin. In the latter case CM-Sephadex was substituted for DEAE-cellulose in the chromatographic columns. It was found that the radioactivity was much less easily removed from hemoglobin than from albumin by treatment with glutathione.

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- Jocelyn, P. C. *Biochemistry of the SH Group*, Academic, New York and London 1972, p. 123.
- Modig, H. G., Edgren, M. and Révész, L. *Int. J. Radiat. Biol.* **22** (1971) 257.
- Sanner, T. and Pihl, A. *Scand. J. Clin. Lab. Invest. Suppl.* **106** (1969) 53.
- Horváth, M., Fóris, G., Cságyoly, E., Sztanyik, L. and Dalos, B. *Int. J. Radiat. Biol.* **21** (1972) 263.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
- Patterson, M. S. and Greene, R. C. *Anal. Chem.* **37** (1965) 854.

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Asymmetric Synthesis of (+)-Diethyl Citramalate

SVANTE BRANDÄNGE, STAFFAN JOSEPHSON and STAFFAN VALLÉN

Department of Organic Chemistry, University of Stockholm, Sandåsgatan 2, S-113 27 Stockholm, Sweden

Two hydroxyacids, 2-isobutylmalic acid¹ and 2-benzylmalic acid,² have been found in optically active forms as components in *Orchidaceae* alkaloids. (+)-2-Methylmalic acid, (+)-citramalic acid, is known to have the (*S*)-configuration.³ Asymmetric syn-