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The quantitation of biotinylated compounds by a solid-phase assay using a ^{125}I-labelled biotin derivative

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The biotin analogue biotinylglycyltyrosine has been synthesized and labelled to a specific activity of 2000 Ci/mmol with ^{125}I. This analogue has been used in conjunction with immobilized streptavidin in an assay which detects as little as 1 fmol biotin or biotinylated molecules in solution. The determination of biotinylated insulin in a tissue extract and the quantitation of a transcription assay are given as examples.

Biotin; Biotinylglycyltyrosine; Streptavidin-Sepharose; ^{125}I labeling; Biotin analog; Biotinyl-UTP; Biotinylinsulin

1. INTRODUCTION

We found it necessary to assay for femtomolar amounts of biotinylated compounds in solution. Although ^{125}I labelled avidin and streptavidin are frequently used as probes for the presence of biotinylated molecules, particularly after Western blotting, no routine quantitative assay is available for the detection of sub-picomolar amounts of biotinylated molecules in solution. Fluorimetric methods detect at most 100 pmol in solution [1], and are not always useful for detecting biotin derivatives. ^{14}C- or ^{3}H-labelled biotin isotope dilution assays can detect at best 1 pmol of unlabelled biotin [2,3]. Charcoal adsorption assays [4] are not useful for detecting biotinylated molecules which are likely to be adsorbed by charcoal. Solid-phase assays using immobilized avidin and ^{3}H- or ^{14}C-labelled biotin have been described [5,6] but their sensitivity is limited to picomolar quantities.

Reliable detection of femtomolar amounts of biotin using a solid-phase assay requires radioactive biotin with a specific activity of at least 1000 Ci/mmol. We found the cost of a commercially available ^{125}I iodinated derivative of biotin prohibitive for use in a routine assay. ^{125}I labelling of biotin using the Bolton-Hunter reagent has also been described [4]. We describe an inexpensive, easily prepared biotin derivative labelled to high specific activity with Na^{125}I. This derivative in conjunction with immobilized streptavidin allows detection of as little as 1 fmol biotin, biotinylated protein or biotinylated nucleotide in solution. As examples of the application of this assay, the determination of biotinylated insulin in a tissue extract and the quantitation of a transcription assay are given.

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2. MATERIALS AND METHODS

2.1. *Biotinylation*

Biotin *N*-hydroxysuccinimide ester (BNHS) was synthesized essentially according to May et al. [7]. From this biotinylglycyltyrosine was prepared as follows: 5 μmol glycyltyrosine (Sigma) was dissolved in 200 μl dimethylformamide containing 10 mM *N*-ethylmorpholine and added to 5 μmol BNHS in the same solution. The mixture was left to react at room temperature for 1 h and then sub-

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jected to high pressure liquid chromatography (HPLC) using a Millipore-Waters HPLC and a μBondapak C-18 Radial Pak column. A 0–70% gradient of acetonitrile in 0.05% trifluoroacetic acid (TFA) was used for elution.

Nα-t-boc Gly^{A1}, *Nε t*-boc Lys^{B29} insulin (Diboc insulin), prepared according to Grant and Von Holt [8] was biotinylated using BNHS essentially according to the method of Hofmann et al. [9]. A small amount of [^{14}C]BNHS was included in the reaction to facilitate the identification of biotinylated insulin on the HPLC. The *t*-boc groups were removed with anhydrous TFA and the product purified by HPLC using a 0–70% gradient of acetonitrile in TFA as described before [8]. The degree of substitution of insulin with biotin was determined by the method of Swack et al. [5] using streptavidin-Sepharose and [^{14}C]biotin. The molar ratio of biotin to insulin in the final product was 0.96:1. 5-(Allylamidobiotin)-UTP was synthesized according to Langer et al. [10].

2.2. *Radioiodination of biotinylglycyltyrosine (BGT)*

BGT was dissolved in 50 μl of 0.01 M phosphate buffer, pH 7.5, and iodinated with ^{125}I by incubation with Na^{125}I (Amersham, 2000 Ci/mmol) in the presence of an Iodobead (Pierce). After incubation, unreacted iodine was removed by passing the incubation mixture over a G10 column (0.5 × 10 cm). Material eluting in the outer volume was concentrated and further fractionated by HPLC to remove noniodinated BGT (see section 3).

2.3. *Preparation and standardization of streptavidin-Sepharose 4B*

Streptavidin (Amersham) was linked to Sepharose 4B using CNBr-activated Sepharose 4B (Pharmacia). The number of biotin binding sites per microlitre suspension was determined using [*carbonyl*-^{14}C]biotin (Amersham) of known specific activity. The specificity of binding of biotinylated compounds was checked in a competition assay using unlabelled biotin. In order to facilitate the washing of the solid-phase streptavidin in the binding assay, underivatized Sepharose 4B can be added.

2.4. *Transcription*

A recombinant DNA molecule derived from the sea urchin genome and the pUC derivative 'Blue Scribe' (Vector Cloning Systems) was transcribed using T_7 RNA polymerase according to the instructions supplied by Vector Cloning Systems. All nucleoside triphosphates were present at concentrations of 0.4 mM. In experiments where UTP was replaced by 5-(allylamidobiotin)-UTP, the latter was used at the same concentration. 15 μCi [^{32}P]GTP was added as indicated under results. After transcription 100 μg carrier RNA was added and the RNA precipitated with 10% trichloroacetic acid. The RNA precipitate was subjected to alkaline hydrolysis in 0.2 M KOH for 16 h at 30°C, neutralized and an aliquot assayed for biotinylated UTP.

3. RESULTS AND DISCUSSION

The biotinylglycyltyrosine was isolated by HPLC (fig.1). The product was freeze-dried and stored at −20°C and proved stable for at least 6 months. The binding of BGT to streptavidin was indistinguishable from that of unmodified biotin as measured by fluorescence quenching (fig.2).

After iodination of BGT and removal of unbound ^{125}I, iodinated BGT was fractionated by HPLC (fig.3). The bulk of the radioactivity eluted as a single peak which was well separated from unlabelled BGT. The separation of the iodinated from the noniodinated compound is expected as a result of the hydrophobicity of the former. Over 98% of radioactivity in this peak bound to streptavidin-Sepharose demonstrating the association of the biotinyl residue with the radioactivity. Iodination did not affect the binding of BGT to streptavidin (fig.2). To determine the specific activity of the iodinated BGT, the latter was quantitated with streptavidin-Sepharose standardized with [^{14}C]biotin of known specific activity. A single run on the HPLC routinely produced ^{125}I-labelled BGT with a specific activity equal to that of the input ^{125}I (1000–2000 Ci/mmol). BGT is thus a stable derivative of biotin which may be easily prepared from commercially available reagents and labelled to high specific activity with Na^{125}I.

The ^{125}I-labelled BGT was used in a solid-phase assay to determine small amounts of non-radioactive biotinylated compounds in solution in

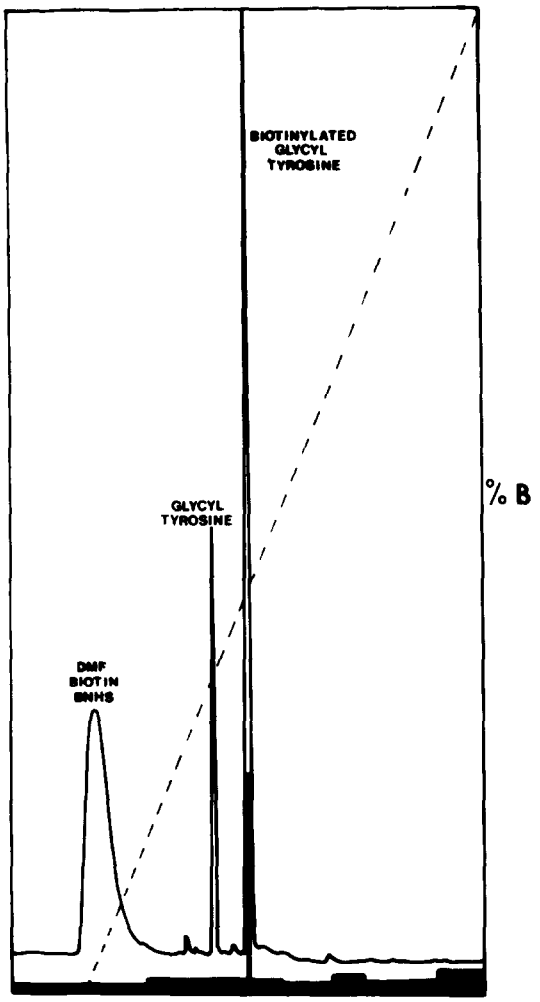

Fig.1. Fractionation of biotinylglycyltyrosine by HPLC. Solution A, 0.05% TFA; solution B, 70% acetonitrile in 0.05% TFA. (– – –) % solution B, (——) A_{230}. To monitor the preparation and isolation of biotinylglycyltyrosine, [^{14}C]biotin was added to some of the preparations and also as a marker in the final HPLC step. The shaded area represents the radioactive profile when [^{14}C]biotin is used in the preparation.

conjunction with a standardized preparation of streptavidin-Sepharose. To this end standardized streptavidin-Sepharose was reacted with the solution containing the non-radioactive biotinylated compound (see also legend to fig.4). After washing the beads, an excess of labelled BGT was added to fill the unoccupied binding sites. The amount of the unknown was calculated from the difference in

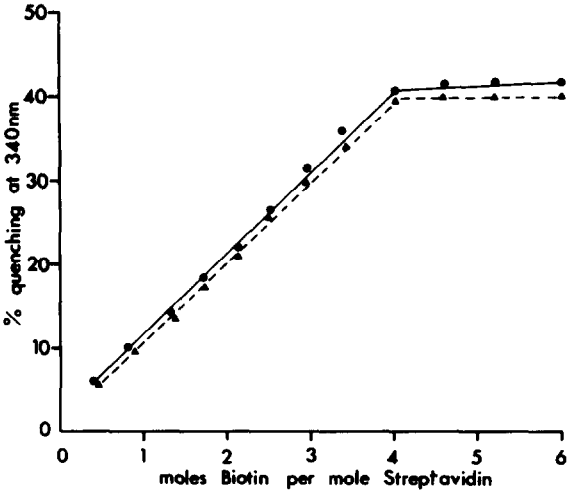

Fig.2. Fluorescence quenching of streptavidin by biotin and BGT. On binding to biotin the fluorescence of the tryptophanyl residues in streptavidin is quenched [1]. 3 ml of a 300 μM solution of streptavidin in 0.01 M phosphate buffer, pH 7.5, were placed in a quartz cuvette and titrated with either biotin or biotin derivatives until no further quenching occurred [1]. Since micromolar quantities of biotin derivatives were used in the assay, iodinated BGT of a specific activity of 0.1 mCi/mmol was prepared to avoid the use of large amounts of radioactivity in the assay. (——) Biotin, (– – –) BGT and iodinated BGT.

binding of iodinated BGT prior to and after exposure of streptavidin-Sepharose to the solution containing the non-radioactive biotinylated compound. Biotinyl Phe-B1-insulin and biotinylated UTP served as model compounds of widely different molecular mass. Both of these compounds bound streptavidin-Sepharose in competition with BGT (fig.4). Neither could be displaced by a 1000-fold excess of ^{125}I-BGT even after incubation for 3–4 h at room temperature. The linearity of competition extended to 95% saturation for biotinyl-UTP and 89% for biotinylinsulin (fig.4). As little as 1 fmol biotinylated insulin or UTP could be detected in solution (fig.4 and table 1). 1 fmol biotin or biotinylated molecule corresponds to a decrease in the order of 3×10^3 dpm ^{125}I-biotinylglycyltyrosine bound to the solid-phase streptavidin depending on the precise specific activity of the ^{125}I-BGT. A 10^6-fold excess of unbiotinylated insulin had no effect on binding (table

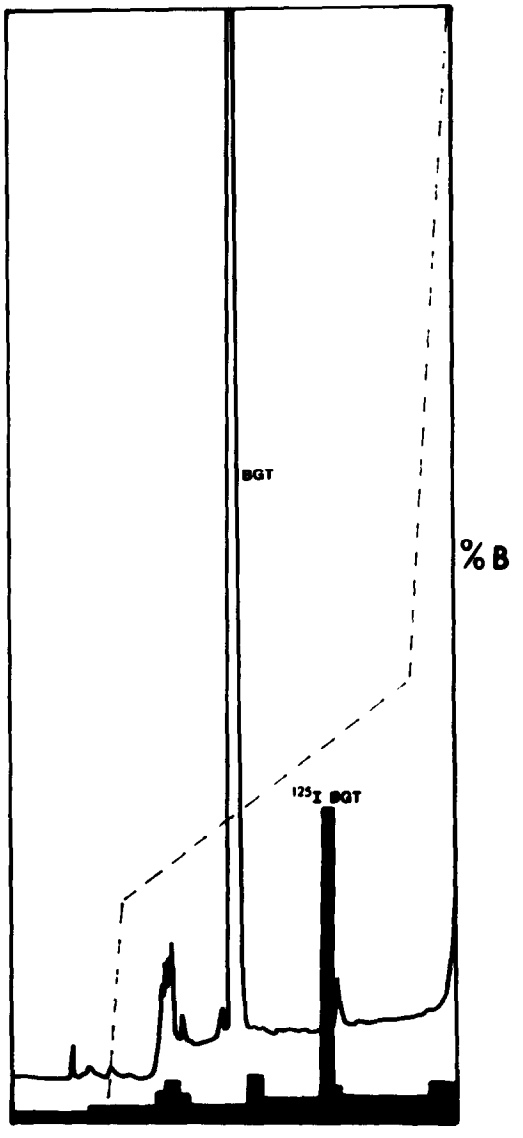

Fig.3. Fractionation of ^{125}I-labelled BGT by HPLC. Elution conditions as in fig.1. (– – –) % solution B, (——) A_{230}. Shaded black area: radioactivity (cpm).

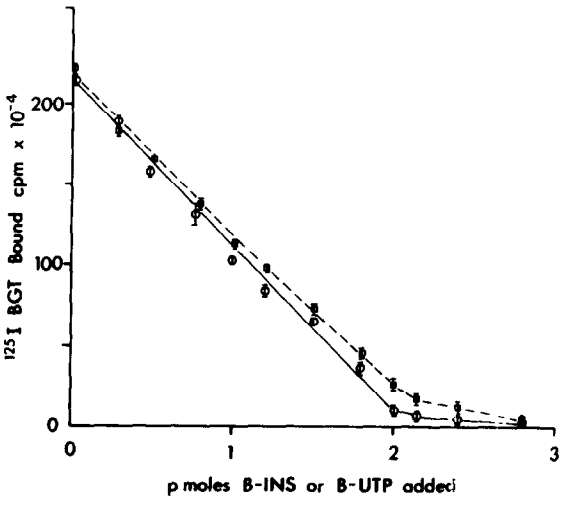

Fig.4. Quantitation of biotinylinsulin and biotinylated UTP binding to streptavidin-Sepharose at the picomolar level. Streptavidin-Sepharose containing 2 pmol biotin binding sites (determined with [^{14}C]biotin) was incubated in 100 μl of 0.1 M phosphate buffer, pH 7.5, with varying amounts of either biotinylinsulin or biotinylated UTP for 30 min at room temperature with shaking. Under these conditions 96% of binding occurred. Excess ^{125}I-BGT was added and incubated for 30 min. The streptavidin-Sepharose was washed and counted. The bars represent the standard deviation. (– – –) Biotinylinsulin, (——) biotinylated UTP.

1). Nonspecific binding of ^{125}I-BGT to streptavidin-Sepharose, was negligible (table 1). Vigorous and repeated washing of the streptavidin-Sepharose did not cause loss of bound material. Usually 3–4 washes sufficed to remove unbound BGT. Binding was not diminished in up to 5% of the nonionic detergent Triton X-100 (table 1). Biotinylated compounds extracted from tissues in the presence of this detergent may thus be assayed without the necessity of detergent removal. Streptavidin-Sepharose has been stored at 4°C for over 2 months without significant loss of binding activity. Re-standardization with [^{14}C]biotin was however routinely performed. The ^{125}I-labelled BGT is stable for weeks at 4°C.

In order to establish whether the assay would be useful for investigating the subcellular distribution of e.g. biotinylated proteins, biotinylinsulin was also assayed for in the presence of rat liver cytosol. 50 fmol could be detected above the background (table 2) caused by the presence of available natural biotin.

The assay has also been used to determine the incorporation of biotinylated UTP into transcribed RNA (table 3). Transcription of DNA from Blue Scribe with T_7-DNA-dependent RNA polymerase in the presence of biotinylated UTP, measured by [α-^{32}P]GTP incorporation, was reduced by 50%

Table 1

Quantitation of femtomolar amounts of biotinylinsulin

Biotinylated insulin added (fmol)	^{125}I BGT bound (cpm)
0	29800 ± 1910
1	27220 ± 1720
5	15820 ± 1260
10	4180 ± 652
10 (after preincubation of streptavidin-Sepharose with 5 nmol biotin)	320 ± 96
5 (+ 5 nmol unlabelled insulin)	14760 ± 1162
5 (in 5% Triton X-100)	16210 ± 1210

Streptavidin-Sepharose with 10 fmol biotin binding sites was incubated with various amounts of biotinylated insulin for 1 h in a total volume of 20 μl with shaking. Sepharose 4B was present as carrier. Under these conditions 84% binding of biotinylinsulin could be achieved. After incubation, 1 μCi of ^{125}I BGT (1500 Ci/mmol) was added and incubation continued for 30 min. The streptavidin-Sepharose was washed with 0.1 M NaCl, 0.01 M Na phosphate, pH 7.5, until the supernatant contained less than 1% of the counts in the pellet. Results represent the average of at least 3 determinations and include the standard deviation

Table 2

Detection of biotinylated insulin in rat liver cytosol

Biotinylinsulin added (fmol)	^{125}I BGT bound (cpm × 10^{-3})	Biotinyl-insulin recovery (fmol)
0 (no cytosol present)	3120 ± 125	
0 (+ 100 μl cytosol)	2062 ± 77	
50	1922 ± 61	47
100	1748 ± 48	101
200	1486 ± 47	187
700	148 ± 21	653

Rat liver cytosol was isolated according to Smith and Von Holt [12]. Streptavidin-Sepharose containing 1 pmol biotin binding sites was incubated with 100 μl of a cytosol preparation (2 mg liver tissue), plus increasing amounts of biotinylinsulin for 1 h at 4°C. After incubation, 1 μCi of ^{125}I BGT was added and the procedure followed as described in the legend to table 1. Results represent the average of three determinations and include the standard deviation

Table 3

Assay for biotinyl-UTP incorporated into transcribed RNA

Incubation time (min)	dpm (from [α-^{32}P]GTP)		pmol
	Control	UTP replaced by biotinylated UTP	Biotinyl-UTP incorporated
0	2786	923	0.72
10	15092	6520	28.64

(table 3) similar to results from other laboratories [10]. Given the specific activity of ^{125}I-biotinylglycyltyrosine, incorporation of femtomolar amounts of biotinyl-UTP can be measured. The assay should also be applicable to biotinylated RNA or DNA probes and offers an alternative to the detection of biotinylated RNA and DNA after immobilization on nitrocellulose. For the preparation of biotinylated hybridization probes, longer linker groups between UTP and the biotinyl residue are required [11].

This assay to quantitate in the femto–picomolar range, biotinylated compounds, including proteins and nucleotides, in solution lends itself to the investigation of the intracellular distribution and the binding of e.g. peptide hormones and other compounds to subcellular structures. The sensitivity of the assay is comparable to that achieved when proteins are labelled with carrier free iodine. The assay therefore makes biotin a useful alternative label for the investigation of molecules which either lose their biological activity when iodinated or lack an aromatic residue as substrate for iodination. The assay will also allow the determination of the specific activities of biotinylated RNA and DNA probes.

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