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COMPARATIVE LABELLING OF α_1-ACID GLYCOPROTEIN BY (^{3}H)-BOROHYDRIDE OR (^{125}I)-IODIDE FOR USE IN A RADIOIMMUNOASSAY

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SUMMARY The labelling of α_1-acid glycoprotein (AGP) with (^{3}H)-sodium borohydride was compared to the labelling with (^{125}I)-sodium iodide by the chloramine T method in view to its use in a radioimmunoassay. The tritium labelling allowed to reach a high specific radioactivity similar to that obtained with iodide ((^{3}H)-AGP : 29.8 mCi/mg ; (^{125}I)-AGP : 30.5 mCi/mg). Each mole of sialic acid residue of AGP contains one atom of tritium. The stability of (^{3}H)-AGP was better than that of (^{125}I)-AGP as indicated by its immunoreactivity as a function of time. Immunoreactivities and standard curves were similar for the two tracers but affinity of antiserum was higher for (^{125}I)-AGP than for (^{3}H)-AGP. Tritium labelling by (^{3}H)-borohydride will be very useful for glycoprotein antigens which cannot be labelled with (^{125}I)-iodide.

INTRODUCTION (^{125}I)-iodide labelling of proteins for radioimmunoassays displays several advantages : low energy γ radiation, 60 days period, high specific activity (2100 Ci/mmol) and high counting yield. The high specific activity obtained with (^{125}I)-labelling allows the detection of femtomolar antigen concentrations. However, the iodination method involves the presence of tyrosyl or histidyl residues in the protein molecule, which limits its use as a general labelling technic. Indeed, the low tyrosine content of some proteins, like mucous glycoproteic antigens and/or the steric hindrance of the carbohydrate residues (1,2) prevent the use of iodination as a general method. The covalent binding of iodinated tyrosyl residues, which increases (^{125}I)-labelling, does not improve the radioimmunoassay sensitivity. Moreover, the carbohydrate residues of these glycoproteins could also be oxidized by chloramine T. Previous papers from VAITUKAITIS (3,4) reported tritium labelling of sialylated glycoprotein hormones by this process but with low specific radioactivity.

We reported in this paper the high specific radioactivity labelling of glycoprotein carbohydrate residues with (^{3}H)-sodium borohydride and we compared this method with the more classical iodination labelling.

MATERIALS AND METHODS (^{125}I)-sodium iodide (220-230 mCi/ml) from the CEA (France) was tenfold diluted with 0.06 M phosphate buffer (pH 7.4) and stored at +4°C. (^{3}H)-sodium borohydride (5-20 Ci/mmol) was from CEA (France).

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α_1-acid glycoprotein (AGP) was isolated from human serum according to Steinbuch et al (5). Its purity was checked by polyacrylamide gel electrophoresis, analytical ultracentrifugation and electroimmunodiffusion using anti-human serum proteins or specific anti-AGP antiserum (Behringwerke).

(^{125}I)-Labelling of AGP and purification of the labelled molecule : AGP labelling was carried out by the chloramine T method (4) : 5 µl AGP (1 mg/ml) was mixed with 10 µl (^{125}I)-INa (20 mCi/ml) and 20 µl chloramine T (2 mg/ml). After one minute stirring, the reaction was stopped by addition of 20 µl sodium metabisulphite (2 mg/ml) and 10 µl potassium iodide (1 mg/ml). Labelled AGP was purified by preparative gel electrophoresis on 7.5 % polyacrylamide in 0.1 M Tris-HCl buffer (pH 8.4) with 5 mA per tube during 4 hours (Fig. 1).

(^{3}H)-Labelling of AGP with (^{3}H)-$NaBH_4$ and purification of the labelled molecule : The labelling was achieved in two steps : a mild periodate oxidation of carbohydrate residues followed by (^{3}H)-$NaBH_4$ reduction of the resulting aldehyde groups. Labelling yield was maximum under the following conditions : 0.1 ml 10 mM $NaIO_4$ was added at +4°C to 10 µg AGP in 1 ml 0.1 M sodium acetate buffer (pH 5) containing 0.15 M NaCl. Oxidation was allowed to proceed for 3 h at +4°C. We did not stopped the periodate oxidation by addition of glucose, as usually done, because under these conditions we found a reduced labelling yield; we prefered to remove $NaIO_4$ by dialysing the solution (24 h at +4°C under stirring) against 0.1 M phosphate buffer (pH 8.5) containing 0.15 M NaCl. The reduction was then carried out by adding 0.5 ml 0.4 M (^{3}H)-$NaBH_4$ (12.5 mCi) in 0.1 M NaOH at room temperature and

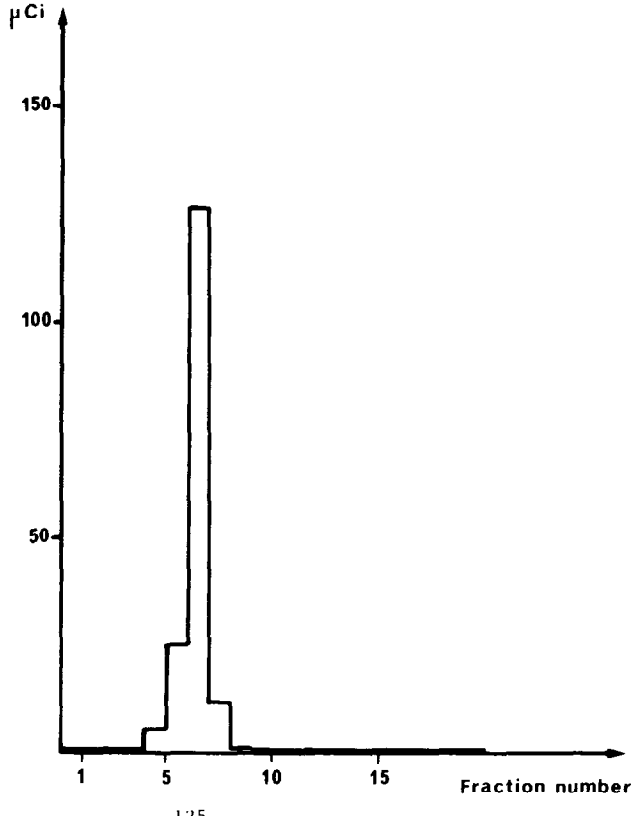

Fig. 1 Purification of (^{125}I)-AGP on polyacrylamide gel electrophoresis : 7.5 % polyacrylamide in 0.1 M Tris-HCl pH 8.4 with 5 mA per tube during 4 hours. Radioactivity of the fractions was measured with an activimeter.

2 h stirring. Labelled AGP was immediately purified by chromatography on Sephadex G-75 at +4°C in 0.15 M NaCl.

Specific activities of labelled AGP :
The specific activity of (^{125}I)-AGP was determined through the electrophoretic plots. That of (^{3}H)-AGP was estimated by radioimmunoassay using (^{125}I)-AGP as label.

Evaluation of the immunoreactivities of purified (^{3}H)-AGP and (^{125}I)-AGP by antiserum titration : The two labelled molecules were diluted with 0.2 M borate buffer (pH 8.4) in order to obtain 15000 dpm per 0.1 ml. Immunoreactivities were determined by double-antibody technique. 0.1 ml rabbit anti-AGP antiserum, with increasing dilutions, and 0.1 ml labelled AGP solution, were added to 0.1 ml 0.2 M borate buffer (pH 8.4) containing 0.1 % albumin (SAB buffer) in polystyrene tubes ; control tubes contained no immuneserum. Determinations were made in triplicate. After 16 h incubation at room temperature, 0.5 ml sheep anti-rabbit γ-globulin immunosorbent (DASP, Organon Tecknika), diluted 10 to 40 times with respect to rabbit antiserum dilutions, was added to each tube. After 4 h stirring at room temperature, the excess of antigen was removed from the antigen-antibody complex by centrifugation (10 min, 2000 g) and two washings with 0.15 M NaCl. The radioactivity of bound (^{125}I)-antigen was directly measured in the pellet using a γ-scintillation spectrometer (Packard Instr.). The (^{3}H)-antigen-antibody complex was transferred in counting vials, rinced twice with 0.3 ml 0.15 M NaCl and solubilized by overnight incubation at 37°C with Soluene-Isopropanol (1:1 ; v/v); 10 ml scintillation liquid (Instagel-1N HCl-H_2O ; 50:3:3, v/v) was added in each vial. A liquid scintillation spectrometer (Packard Tri-Carb) was used to measure the radioactivity.

Standard curves of the radioimmunoassay :
We used the previously described procedure to measure the competition between the two labelled antigens and the cold antigen, but instead of 0.1 ml SAB buffer, we

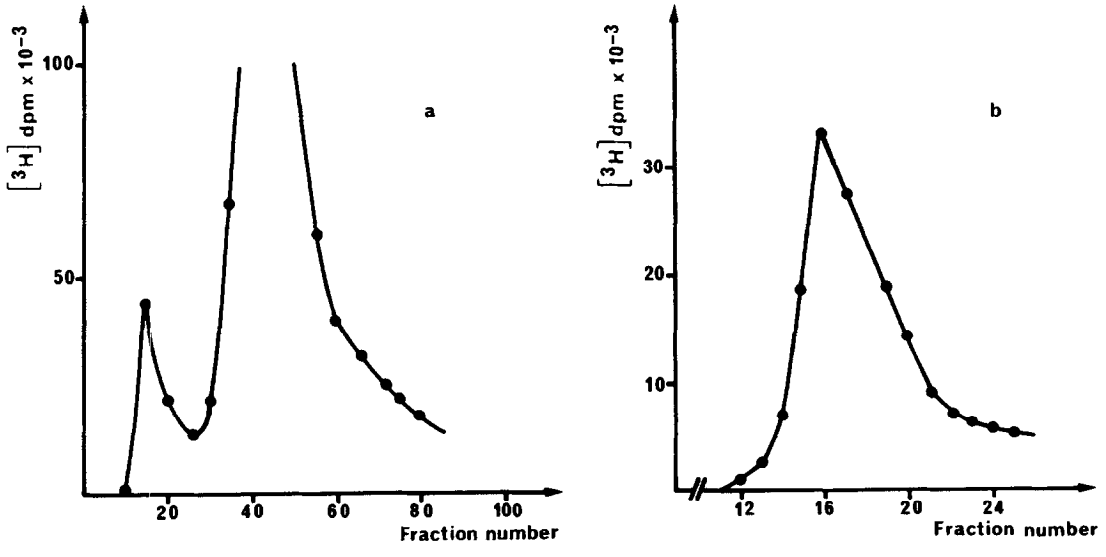

Fig. 2 Purification of (^{3}H)-AGP : 10 µg of (^{3}H)-AGP are purified on sephadex G-75 column (1.6 x 52 cm) in distillated water at +4°C with a rate of 0.2 ml/min. Each fraction contains 2.2 ml.
a - represents the total diagram
b - represents the first peak of the total diagram. The second one corresponds to (^{3}H)-$NaBH_4$ free. Radioactivity was measured by liquid scintillation.

added 0.1 ml solution of cold antigen in SAB buffer with increasing dilutions ranging from 6.25 to 200 ng per ml. The dilution of rabbit anti-AGP antiserum was 1:2000 and that of DASP 1:20. (^{125}I)-AGP and (^{3}H)-AGP concentrations were the same as previously. All antiserum dilutions are indicated as final dilutions.

RESULTS

1- (^{125}I)-Labelling : Factors affecting the (^{125}I)-labelling of AGP were earlier described (7). The results of 5 AGP labellings are reported in table I. The labelling yield was related to chloramine T concentrations and to the ratio (^{125}I)/protein. The best result was obtained in the second experiment.

2- (^{3}H)-$NaBH_4$ labelling :

. Periodate oxidation time and reduction time

The ratios of periodate moles or borohydride moles towards carbohydrate moles being constants, i.e. periodate/carbohydrates = 50 and borohydride/carbohydrates = 0.5, we varied oxidation time and reduction time.

Table I. (^{125}I)-Labelling : Specific radioactivities and yield of labelling for AGP are reported in different experimental conditions.

Sample	Chloramine T (µg)	AGP (µg)	(^{125}I) (µCi)	Specific activity (mCi/mg)	Yield (%)
1	40	5	200	28.5	71
2	40	5	200	30.5	76
3	20	5	200	20.8	52
4	20	3	200	24.2	37
5	40	5	200	25.5	64

Table II. (^{3}H)-Labelling : Influence of periodic oxidation or reduction time course on the specific radioactivity of the tracer.

Sample	Periodic oxidation (hours)	Reduction (hours)	Specific radioactivity (mCi/mg)
1	1	2	0.103
2	4	2	0.311
3	3	1/2	0.103
4	3	1	0.306
5	3	3 1/2	0.201

Table III. (^{3}H)-Labelling : Influence of (^{3}H)-$NaBH_4$ concentrations on the specific radioactivity of the tracer and the yield of labelling.

Sample	Radioactivity introduced (μCi)	Specific radioactivity (mCi/mg)	Yield (%)
1	5	0.587	29
2	50	0.729	15
3	2000	1.77	0.9
4	4000	9.42	2.3
5	12500	29.8	2.4

Table II shows that specific activity increased with increasing oxidation time, while reduction time had apparently no effect.

. Relationship between (^{3}H)-$NaBH_4$ concentrations and the specific activity of the labelled molecule

We have studied the labelling efficiency when increasing (^{3}H)-$NaBH_4$ concentrations in the reaction mixture (Table III).

Above 2 mCi (^{3}H)-$NaBH_4$ the specific activity of (^{3}H)-AGP was directly proportional to the borohydride concentration. Indeed, reduction was carried out with commercia (^{3}H)-$NaBH_4$, without addition of cold borohydride and the increase in (^{3}H)-$NaBH_4$ specific activity was parallel with an increase in reducing capacity, which probably explains the low specific activity increase between experiment 1 and 2 while 10-fold more radioactivity was added in the later.

Under optimized labelling conditions, (^{3}H)-AGP showed a specific activity of 29.8 mCi/mg. With a molecular weight of 44000 daltons, one AGP mole contained 13.10^5 Ci, i.e. 45 tritium atoms.

. (^{3}H) Distribution in the (^{3}H)-AGP

Sialyl residues on (^{3}H)-AGP were specifically hydrolyzed by 1 h treatment with 0.1 N H_2SO_4 at 80°C. The hydrolysate was chromatographied on a sephadex G-10 column (0.6x40 cm) and radioactivity of the eluates was determined.

The delayed fraction which corresponds to the (^{3}H)-sialyl residues, accounts for 32 % of the layered radioactivity. Therefore, 1/3 of the tritium atoms included in the AGP molecule was associated with the sialyl residues. As one mole of AGP contains 15 moles of sialic acid, each mole of this residue contains one atom of tritium.

3- Stability of the labelled molecules : The stability of (^{3}H)-AGP was estimated by its immunoreactivity in function of time towards anti-AGP antiserum diluted at 1:2000. The bound radioactivity shows an exponential decrease with

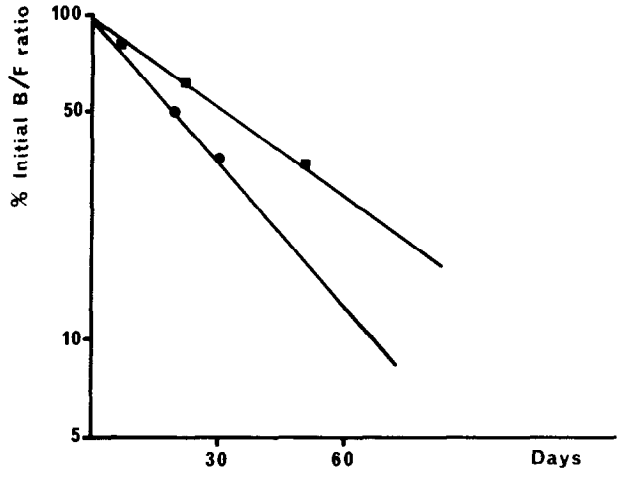

Fig. 3 Stability of the labelled molecules : Binding of trace labelled antigens (10000 cpm per 0.1 ml buffer) to 1:2000 diluted antiserum (0.1 ml) was reported to the initial binding and plotted as a function of time of storage. Incubation in 0.2 M borate buffer pH 8.4 containing 0.1 % bovine serum albumin was carried out in triplicate overnight at ambiant temperature. Separation was made with a double-antibody as indicated in the text.

■ (^{3}H)-AGP
● (^{125}I)-AGP

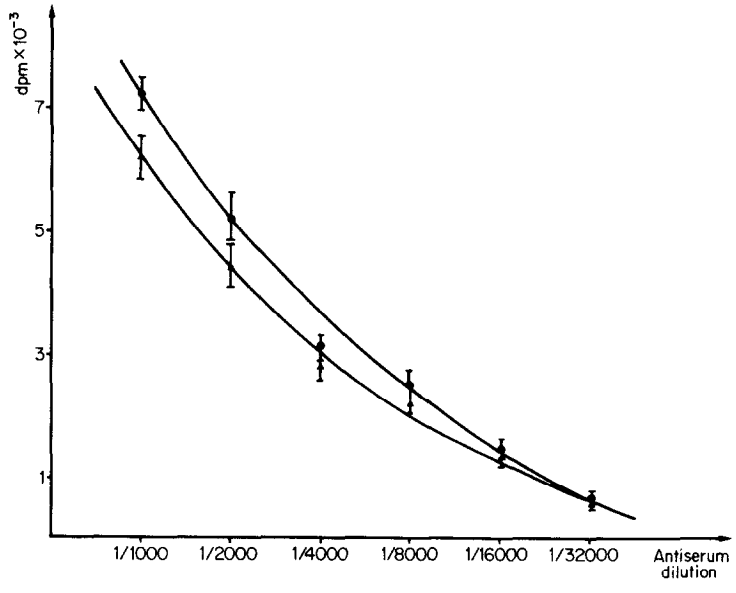

Fig. 4 Titration curves of the antiserum : Bound radioactivities of trace labelled antigens (10000 cpm) were plotted as a function of anti-AGP final antiserum dilutions from 1:1000 to 1:32000. Incubation conditions were the same as in Fig. 3

● (^{3}H)-AGP
▲ (^{125}I)-AGP

a period of 35 days (Fig. 3). The stability of (^{125}I)-AGP, measured in the same conditions, was not so good. The exponential decrease reaches 50 % after a 22 days period.

4- Comparison of the immunoreactivities between (^{3}H)-AGP and (^{125}I)-AGP :

. Titration of the antiserum

Radioactivities bound to the anti-AGP antiserum as a function of its dilution are plotted in Fig. 4. Whatever the dilution may be, the bound radioactivity values are nearly the same for the both labelled molecules. The slopes of the curves are not superposable. According to the Berson's criteria (8), the antiserum affinity was slightly higher for (^{125}I)-AGP than for (^{3}H)-AGP. The similar values of the bound radioactivities agree with the specific activities reported before.

. Standard curves

The slopes for the standard curves of (^{125}I)-AGP and (^{3}H)-AGP were identical as shown in Fig. 5. This means that immunoreactivity against anti-AGP antiserum of the two labelled molecules are similar. The (^{3}H)-AGP curve was slightly shifted to the higher standard values, owing to the difference between the antiserum affinity for the labels.

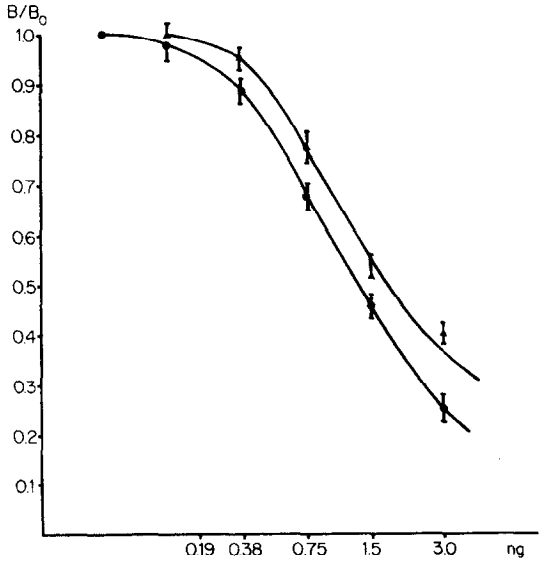

Fig. 5 Standard curves of radioimmunoassays : Competitive binding between labelled antigens (10000 cpm) and cold antigen (0.1 ml) in the concentration range from 6.25 to 200 ng per ml was carried out with 0.1 ml 1:2000 diluted anti-AGP antiserum in the same experimental conditions as in Fig. 3. B_0 is the bound radioactivity in the absence of cold antigen.
▲ (^{3}H)-AGP
● (^{125}I)-AGP

DISCUSSION

The disadvantages of (^{125}I)-labelling of glycoproteins (steric alterations of the protein, denaturation by oxidation during the labelling) can be avoided by labelling the carbohydrate residues with (^{3}H)-$NaBH_4$.

In radioimmunoassay, the labelled antigen must contain a specific activity high enough to allow the detection of femtomoles of cold antigen. The yield of AGP labelling by (^{3}H)-$NaBH_4$ depends on several factors : periodate oxidation time, reducing capacity of borohydride and specific activity of (^{3}H)-$NaBH_4$ which correspond to 0.7 tritium atom in one mole of borohydride. Under optimized conditions, the specific activities of (^{3}H)-AGP and (^{125}I)-AGP were similar. One can calculate that 70 tritium atoms must be introduced in one mole of AGP to obtain the same specific radioactivity as that resulting from binding of 1 (^{125}I) atom. These activities correspond statistically to the binding of 45 tritium atoms per mole of AGP and 2 (^{125}I) atoms per 3 moles of AGP.
At higher specific radioactivity, (^{125}I)-AGP and (^{3}H)-AGP were readily denaturated as evidenced by immunoreactivity decrease which was higher for (^{125}I)-AGP than for (^{3}H)-AGP. The slightly lower affinity of the antiserum for (^{3}H)-AGP as compared to (^{125}I)-AGP, had no effect on the sensibility of the radioimmunoassay which was similar for both labelled glycoproteins.

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