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EFFECT OF THE SPECIFIC ACTIVITY OF THE TRACER ON RAT LUTEINIZING HORMONE RADIOIMMUNOASSAY

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Rat luteinizing hormone /LH/ was labelled with ^{125}I by the Chloramine T method. ^{125}I-LH, used as tracer in radioimmunoassay, was separated from the labelling reaction mixture by gel filtration. By using the proper protein/radioiodine ratio in the labelling reaction mixture the specific activity of ^{125}I-LH was adjusted to 2.5-20.5 MBq μg^{-1}. The influence of the specific activity on the assay parameters as well as on the tracer stability was investigated.

The specific activity of the tracer /expressed as MBq μg^{-1} or MBq $mmol^{-1}$/ can drastically influence the dose response curve of the radioimmunoassay. The ratio of concentration or quantity of analyte /antigen/ bound /B/ and unbound /F/ to antibody is given by /1/:

$$\frac{B}{F} = \frac{q}{p \cdot \frac{1}{1 + B/F} + \frac{1}{K}} \qquad /1/$$

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where q stands for the concentration of the antibody, p for the concentration of the analyte /antigen/ and K is the stability constant of the antibody-antigen complex:

$$K = \frac{/QP/}{/Q/\ /P/} \qquad /2/$$

where /Q/ stands for the concentration of the antibody, /P/ for that of the antigen /analyte/ and /QP/ for the concentration of the antibody-antigen complex.

It should be noted that the term p comprises the concentration of the analyte /p_a/ and that of the tracer /p^*/, i.e.,

$$p = p_a + p^* \qquad /3/$$

Thus at zero analyte concentration $p = p^*$.

It is evident from Eq. /1/ that at given q and K and zero analyte concentration the B/F ratio increases with decreasing p^*. From this point of view the specific activity of the tracer /P^*/ should be as high as possible. In the case of e.g. rat prolactin, which exhibits 7 tyrosine residues, 14 ^{125}I atoms could be introduced per molecule into positions 3 and 5. On the other hand, the increase of the specific activity, i.e., the increase of the number of radioiodine atoms per molecule deteriorates the immunoreactivity of the tracer. In addition, the higher the specific activity, the higher the rate of decomposition of the tracer molecule due to the "decay catastrophe". Generally the compromise is accepted that not more than one radioiodine atom is introduced per molecule of tracer. The number of radioiodine atoms introduced into the tracer molecule can be calculated from the starting molar ratio of the reactants in the labelling mixture taking into account the efficiency of the label-

ling as well. The substitution degree thus calculated is an average, which is seldom a whole number if the protein to be labelled exhibits more than one residue suitable for substitution.

If M stands for the maximum number of reactable residues per molecule, N for the number of residues actually reacted, R = I/P for the number of radioiodine atoms introduced per mole of protein, F_n, i.e., the fraction of radioactivity in the tracer contributed by molecules containing N radioiodine atoms can be calculated as follows /Ref 2/:

$$F_n = \frac{M!\ N}{N!\ (M-N)!\ R} \left(\frac{R}{M}\right)^N \left(1-\frac{R}{M}\right)^{M-N} \qquad /4/$$

The distribution of the mono-, poly- and unlabelled proteins as a function of the iodine/protein ratio /R/ was demonstrated in one of our previous papers[3].

Actually the distribution of mono-, poly- and unsubstituted peptides is complicated by the fact that the substitution rate of radioiodine may be different in the tyrosine residues, partly due to the steric hindrance caused by the ternary structure. Thus the optimum specific activity of the tracer resulting in high sensitivity and high stability cannot be calculated according to Eq. /4/, instead it can be assessed empirically. The aim of this paper is to report experimental findings concerning the influence of specific activity on the assay parameters as well as on the stability of the tracer.

MATERIALS AND METHODS

Rat luteinizing hormone /rLH/ and rabbit anti rLH serum were kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases /NIAMDD/. The reference preparation was NIAMOD rat LH-RP-3.

The antigen, i.e., NIDDK-rLH-I-7 was labelled with ^{125}I by the use of the Chloramine T method. The labelled hormone was stored deep-frozen or lyophilized. In the latter case human serum, horse serum or phosphosaline buffer containing 1% bovine serum albumin /Merck/ were co-lyophilized with ^{125}I-rLH.

100 µl of ^{125}I-rLH obtained by reconstitution of the lyophilized samples, rLH standard in 100 µl of PBS buffer /pH 7.4/ containing 1.5% gamma globulin and 0.1% BSA and antiserum /initial dilution 1:180000/ in 100 µl assay buffer were dispensed into plastic tubes. The tubes were incubated for 24 h at room temperature.

After incubation 1 ml of precipitating agent /PEG 6000 solution and 2nd antibody/ was added to the tubes, the precipitate was centrifuged, the supernatant aspirated and discarded and the radioactivity of the precipitate counted.

RESULTS

The influence of specific activity on the assay parameters

Immediately after the purification step the rLH tracers of different specific activities were diluted to an activity of about 30000 cmp/100 µl using the assay buffer and tested in radioimmunoassay /Fig. 1/.

It can be seen from Fig. 1 that both the shape of the dose-response curve and the zero-binding are highly influenced by the specific activity of the tracer. After the test a part of the tracers was aliquoted into plastic tubes without dilution and stored at -20 °C. The other part was properly diluted and freeze-dried. Human serum, horse serum and phosphosaline buffer containing 1% BSA were used for the dilution. Ten days

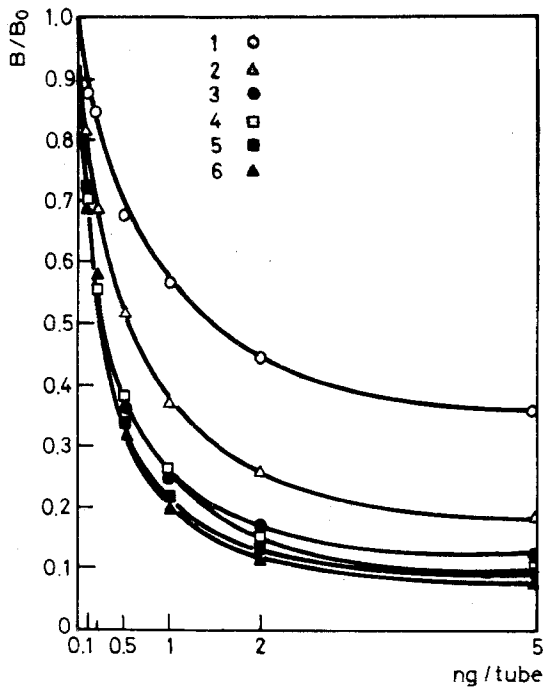

Fig. 1. Dose-response curves obtained with tracer of different specific activities
o - 1.6 MBq μg^{-1}, B_o/T: 0.12; Δ - 2.8 MBq μg^{-1}, B_o/T: 0.22; • - 5.6 MBq μg^{-1}, B_o/T: 0.33; □ - 9.0 MBq μg^{-1}, B_o/T: 0.37; ■ - 13.6 MBq μg^{-1}, B_o/T: 0.42; ▲ - 19.0 MBq μg^{-1}, B_o/T: 0.45

later the tracers were tested for binding characteristics /Fig. 2/.

It can be seen that the storage conditions have no effect on the shape of the dose-response curve. The same result has been obtained with the other tracers both of higher and lower specific activities.

Table 1 shows the values of B_o/T obtained using tracers of different specific activities.

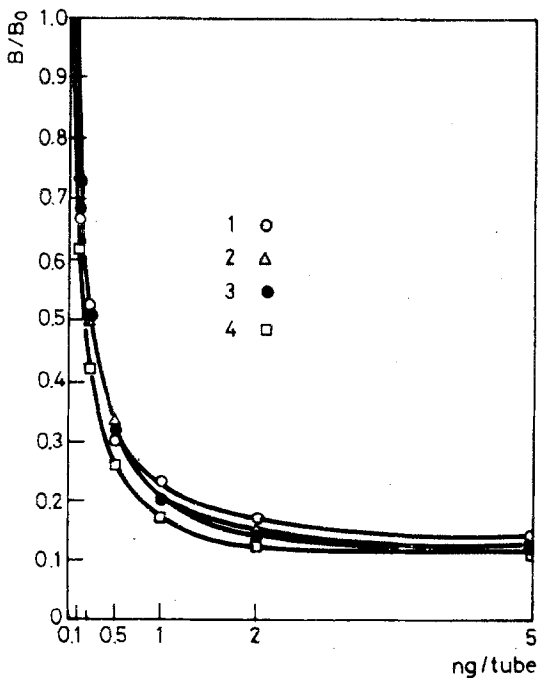

Fig. 2. Dose-response curves obtained with tracers /9.0 MBq μg^{-1}/ stored deep-frozen or freeze-dried for 10 d.
The zero-binding of tracers co-lyophilized with: o - human serum 0.31, Δ - horse serum 0.30, • - Buffer 0.29. Stored deep-frozen: □ - 0.31

The influence of specific activity on the stability of tracers

The freeze-dried and frozen tracers were stored for 6 weeks and tested every seventh day.

Figures 3-5 show the linear regression analysis of the data obtained by the stability test of the tracers co-lyophilized with human serum, assay buffer and stored frozen, respectively.

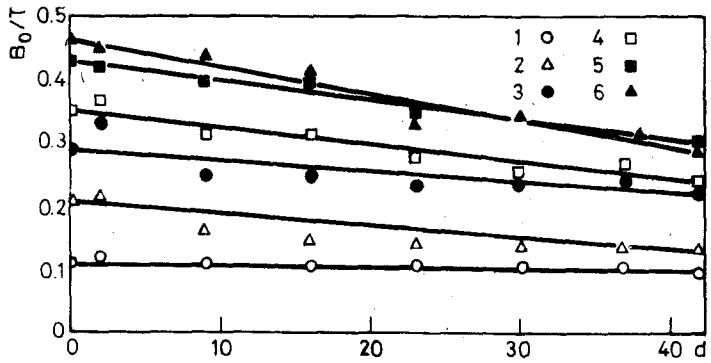

Fig. 3. The effect of specific activity on the stybility of the tracers stored co-lyophilized with human serum
○ - 1.6 MBq μg^{-1}, I/P: 0.5; Δ - 2.8 MBq μg^{-1}, I/P: 0.9; ● - 5.6 MBq μg^{-1}, I/P: 1.8; □ - 9.0 MBq μg^{-1}, I/P: 2.9; ■ - 13.6 MBq μg^{-1}, I/P: 4.3; ▲ - 19.0 MBq μg^{-1}, I/P: 6.0

TABLE 1

The values of B_0/T after 10 days of storage

MBq μg^{-1}	Tracers co-lyophilized with			Tracers stored deep-frozen
	Human serum	Horse serum	Buffer	
1.6	0.11	0.08	0.10	0.10
2.8	0.16	0.14	0.16	0.16
5.6	0.25	0.24	0.25	0.30
9.0	0.31	0.30	0.29	0.31
13.6	0.40	0.42	0.42	0.41
19.0	0.43	0.40	0.40	0.39

DISCUSSION

Assuming that the radioactivity of the tracer is constant in the assay tubes, it is evident that the tracer concentration is inversely proportional to the

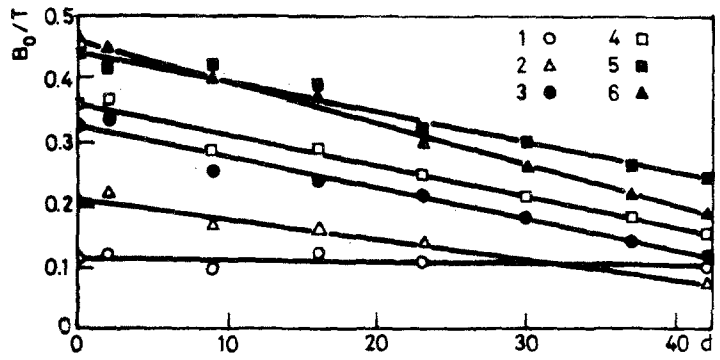

Fig. 4. The effect of specific activity on the stability of the tracers stored co-lyophilized with the assay buffer /Symbols as in Fig. 3/

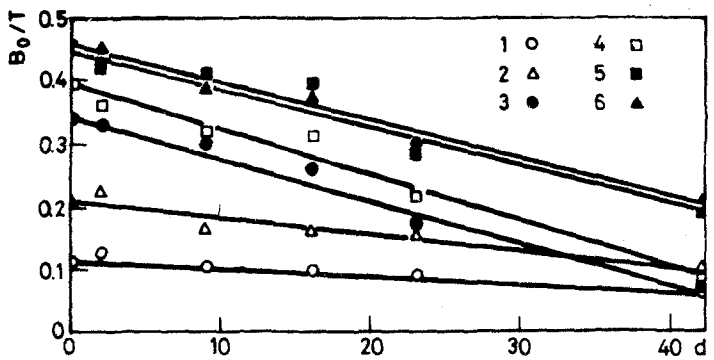

Fig. 5. The effect of specific activity on the stability of the tracers stored deep-frozen /Symbols as in Fig. 3/

specific activity. It is also evident that at zero analyte concentration, i.e., when no unlabelled antigen is present in the incubation mixture, the higher the specific activity the higher is the antibody-bound fraction of the tracer /zero binding/. This is clearly demonstrated by the plots on Figs 3-5.

On the other hand it can be also seen from these plots that the zero binding decreases with the time

elapsed from labelling. This finding can be attributed to the time-dependent radiolytic degradation of radioiodine-labelled peptides.

The rate of radiolytic degradation increases with the increase of radioiodine/protein /I//P/ ratio, i.e., with the number of radioiodine atoms per protein molecule. However, the I/P ratio depends on whether radioiodine or protein is applied in excess in the labelling reaction mixture, it can be shown that when more than one substitution site exists in the protein molecule the labelling results in a mixture of mono- and poly-labelled products. The higher the number of substitution sites per molecule, the greater is the variety of polylabelled molecules. Taking into account that positions 3 and/or 5 of tyrosyl residues are the predominant substitution sites in peptides, no uniformly labelled peptide can be produced even in the case when the molecule to be labelled exhibits only a single tyrosyl residue.

The distribution of the mono- and poly-labelled peptides is rendered more complicated by the different reactivities of the tyrosil residues[4].

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