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Radioiodine Labelling of Insulin Using Dimethylsulfoxide as a Labelling-Aid

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Abstract

Using dimethylsulfoxide (DMSO) as a labelling aid, insulin-^{125}I of radioimmunoassay use has been effectively prepared. A small amount of DMSO was added to usual labelling mixture and the reaction time was controled. The labelled insulin obtained in such a way showed improved bindabilities to the antibody and thus expressed larger dose-gradients in the plots of standard dose-response curves even though the labelling rate was decreased to some extent. However, by extending the reaction time to about 1 min, average labelling yield of 30% could be obtained. The average increase of bindability (B/F) in definite antiserum dilution was 2.5 comparing with 1.5 obtained in the absence of DMSO. Thus, the net bindability increase was 70% of those obtained in the absence of DMSO.

By means of a NMR spectrometry, it has been confirmed that the DMSO in the labelling mixtutre is converted to dimethylsulfone by chloramine-T. The results, generally agreed with the Stagg's postulation, were discussed in view of a competitive oxidation of DMSO with disulfide linkages of the insulin molecule by the chloramine-T.

요 약

디메틸썰폭사이드를 표지보조제로 사용하여 방사면역측정용 ^{125}I 표지인슈린을 효과적으로 만들 수 있었다. 디메틸썰폭사이드 조금을 통상의 표지반응 혼합물에 첨가하여 반응시간을 조절하여 보았더니 표지반응속도는 약간 느렸으나 이렇게 하여 얻은 표지인슈린은 항체와의 결합능이 좋았으며 표준곡선을 점시했을 때 큰 구배(句配)를 보였다. 표지반응수율도 반응시간을 1분으로 늘림에 따라 약 30%로 늘어났다. 평균 항체결합능(B/F)의 증가는 일정항체 희석비율하에서 표지보조제없이 표지한 인슈린을 쓴 때가 1.5인데 반해 표지보조제를 쓴 경우는 2.5로서 약 70%의 향상을 보였다.

핵자기공명분광법으로 표지혼합물에 첨가한 디메틸썰폭사이드는 클로라민티에 의해 디메틸썰폰으로 산화된다는 것을 확인하였다.

Stagg 등의 결과와 대략 같은 이 실험결과를 디메틸썰폭사이드와 인슈린분자 중의 유황결합이 클로라민티에 의해 경쟁적 산화를 받는 것으로 보고 이를 토의하였다.

1. Introduction

Radioiodine labelled insulin is used popularly as a tracer for both radioimmunoassay and in-vitro metabolic studies. As the previously known method of radioiodination of peptide hormones induces quite large amount of biologically and immunologically inactive molecules, many investigators emphasized that the labelled peptide hormones should have intact activities[1,2]. Even though the lactoperoxidase procedure [3], and iodine monochloride procedure[4] are also known, the chloramine-T procedure[5] is still considered to be most popular.

Since introduction of just one radioiodine atom per molecule of the hormone is quite essential for maintaining its biological and immunological activities[6,7], the cause of such deactivations of the labelled hormones may be attributable to the over iodination. However, B.H. Stagg et al[8] postulated that the deactivation of gastrin can be prevented to some extent by modifying the radioiodination procedure; i.e., the DMSO is oxidized competitvely with the gastrin molecule by the chloramine-T when it is in the labelling mixture. Thus, the oxidation of sulfur of the methionyl residue at position 15 in the gastrin can be diminished. Since the sulfur atoms present both in the peptide hormone and DMSO are susceptible to oxidation, the DMSO in the labelling mixture may also be oxidized. B.H. Stagg et al[8]. carried out some iodination experiments using varying amount of chloramine-T, and they insisted that the reduction of biological activity is mainly due to the oxidation of sulfide in the hormone by the chloramine-T, and the over iodination is a minor factor in a small scale, carrier free radioiodination. Further, Morley, J. S.[9] has shown that the methionyl residue at position 15 in the gastrin molecule is readily oxidized to the sulfoxide and sulfone derivatives, and such a process leads to loss of bioloical activity.

B.H. Stagg et al[8]. has also attempted to confirm such oxidation occured by an aminoacid analyses of gastrin after exposing to chloramine-T. The results of aminoacid analyses of gastrin indicated that the methionine was absent and an equivalent amount of methionine-S-oxide was present. Therefore, they used DMSO as a scavenger for protection of the methionyl residue from the oxidative action of the chloramine-T. They observed that the inclusion of DMSO in the iodinations of gastrin with ^{125}I yielded a radioiodinated peptide with full biological activity and without reduction of the specific activity. The immunological activity was reported to be increased showing that the percentage of labelled gastrin to its antibody was increased from 34 to 58.

Even though S. Namba[10] has extended the application of the technique to the lablling of several peptide hormones, it is still wondered that the DMSO is truly oxidized by the chloramine-T. No identification has been made for the oxidation product of DMSO in the labelling mixture by the chloramine-T. Thus, in the present paper, the authors has attempted to identify the oxidation product of DMSO by a NMR spectrometry, and to confirm that the applicability of the tochnique to the routine production of the insulin radioimmunoassay kits.

2. Experimental

2.1. Modified Radioiodination of Insulin

Table 1. Dilution ratio and B/F value of each labelled fraction and labelling batch

DMSO (ul)	Reaction time (sec)	Labelling yield (%)	Dilution ratio to yield 2×10^5 cpm/ml		B/F*		B/F×dilution ratio to yield 2×10^5cpm/ml	
			each fraction	mean	each fraction	mean	each fraction	mean
0	3	12.6	12.3		0.36		4.43	
			24.0		1.07		25.68	
			24.4		2.28		55.63**	
			4.0	16.2	2.31	1.50	9.24	23.70
10	3	9.8	11.2		1.15		12.88	
			20.7		1.94		40.16	
			17.4		3.60		62.64**	
			5.6	13.7	2.32	2.25	12.99	32.16
10	20	20.3	12.6		1.45		18.27	
			40.8		2.15		87.72	
			21.2		3.88		82.26**	
			14.5	22.2	2.24	2.43	32.48	55.18
10	40	25.2	15.1		1.84		27.78	
			45.2		2.26		102.15	
			24.4		3.92		95.65**	
			15.2	25.0	2.14	2.54	32.52	64.53
10	60	29.4	18.8		1.82		34.22	
			50.3		2.15		108.15	
			28.5		4.10		116.85**	
			16.6	28.6	2.03	2.52	33.69	72.23
10	100	27.8	18.0		1.75		31.50	
			48.2		2.30		110.86	
			25.8		3.97		102.43**	
			17.4	27.4	2.13	2.54	37.10	70.40

*the relative values obtained in 3 hrs' incubation at 37°C

**the well labelled fraction in each batch

Radioiodination of insulin was carried out according to the ordinary method proposed by Greenwood, Hunter, and Glover[5] but with slight modifications; i.e., to the ordinary labelling mixture a small amount of DMSO was added before the addition of chloramine-T solution.

Into a 12×70 mm polypropylene vial, 20 ul of 0.5 M phoshate buffer, pH 7.4, 300 uCi of sodium iodide-^{125}I (carrier and reducing agent free for protein iodination, Radiochemical Centre, England), 1 ug of insulin (porcine, recrystallized, 25.5 IU/mg, Schwarz/Mann) were added in sequence. Ten ul of pure DMSO (E, Merck) was added and followed by addition of 10 ul of 0.35% chloramne-T solution. After maintaining definite time intervals, 20 ul of 0.24% solution of sodium metabisulfite was added to stop the reaction, and then 20 ul of blue

plasma was added.

The reaction mixture was applied to the holes of the starch gel plate which was prepared a day before the conduction of lablling. Electrophoresis was carried out in a refrigerator for 4 hrs at the constant voltage of 300 V. The gel plate was removed, and an autoradiography was conducted. The gel which comes under the spot of the developed X-ray film was sliced into about 2 mm width in a horizontal direction using a razor blade. Each slice was put into test tube, respectively, and numbered.

Then they were deeply frozen. Using phosphate buffer, pH 8.6, containing 0.5% bovine serum albumin as an eluate' the lablled hormone was eluted from the gel; 1 ml of the buffer was added to each tube, and gently pressed the gel several times with round tipped Lorex glass bars, and then compressed out the eluate. Such manpulation was repeated three times for all of the tubes (usualIy 4 tubes per batch) using a new 1 ml eluate each time. After measuring the radioactivities for each fraction, they were diluted to the radioactivity concentration of 2×10^5cpm/ml using the same buffer. The radioactivity in aliquot of electrolyte (anode side) was also counted to check the over-all labelling yield according to the following equation;

$$\text{Labelling yield}(\%)=\frac{\sum a_i+\sum b_i}{\sum a_i+\sum b_i+c}\times100 \quad (1)$$

where a, b, and c, are radioactivities (cpm) in eluate, in starch gel, and in total volume of anode side electrolyte, respectively. To assess the bindabilities of the labelled insulin, and to select the well labelled fractions for the insulin RIA, incubation mixtures were made, and incubated them for 24 hrs at 4°C. The labelled insulin bound to its antibody (B) and the free labelled

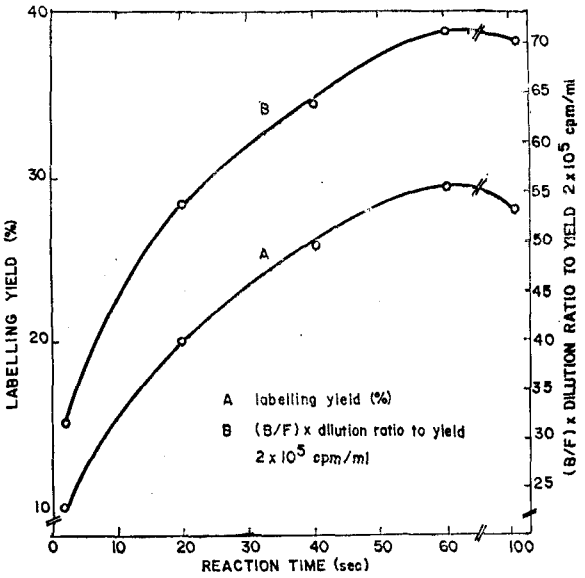

Fig. 1. Dependence of labelling yield, and B/F×dilution ratio on the reaction time in using 10 ul DMSO as a labelling-aid

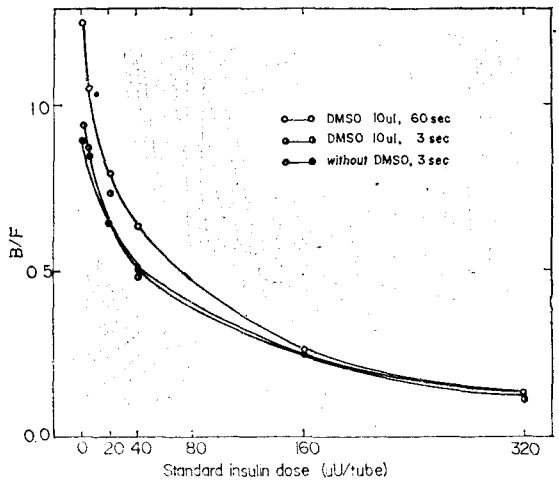

Fig. 2. Standard dose-response curves for insulin radioimmunoassay obtained by using ^{125}I-insulin labelled in various conditions.

insulin (F) were separated by addition of 0.3 ml of dextran coated charcoal suspension,[11,12] agitation and subsequent centrifugation. The radioactivity for the supernatant (antibody bound hormone (B)) and precipitate (free hormone (F)) were sepa-

rately counted. After subtraction of the background activity the B/F ratios were calculated for each fraction according to the following equation;

$$B/F=\frac{B-[(B-F)\times D]}{F} \qquad (2)$$

where D is the ratio between the radioactivity in the supernatant (B) and the total radioactivity ($B+F$) in each control tube. (Table 1), (Fig. 1).

Standard dose-response curve was plotted using the well labelled fractions obtained in various labelling conditions. (Fig. 2).

2.2. Identification of the Oxidation Product of DMSO

NMR spectra for the following samples were obtained using deuterated water as a solvent, and dioxane, TMS, or DSS as a reference standard, respectively.

(1) DMSO
(2) chloramine-T
(3) DMSO plus chloramine-T
(4) dimethylsulfone (the most plausible product)

The dimethylsulfone was synthesized via following reaction scheme;[13]

$$2CH_3I+Na_2S \xrightarrow{\text{reflux 2 hrs}} CH_3SCH_3+2NaI \qquad (3)$$

$$CH_3SCH_3(\text{in HAc})+H_2O_2(\text{excess}) \xrightarrow{\text{reflux}} CH_3SO_2CH_3 \qquad (4)$$

The dimethylsulfide was steam distilled and dried. Then it was treated with an excess hydrogen peroxide in glacial acetic acid to obtain dimethylsolfone. The crude dimethyl sulfone was recrystallized from benzene and petroleum ether. (m.p.; 109-110°C, Ref. 110°C[13]). (Fig. 3).

3. Results and Discussion

3.1. Labelling Yield

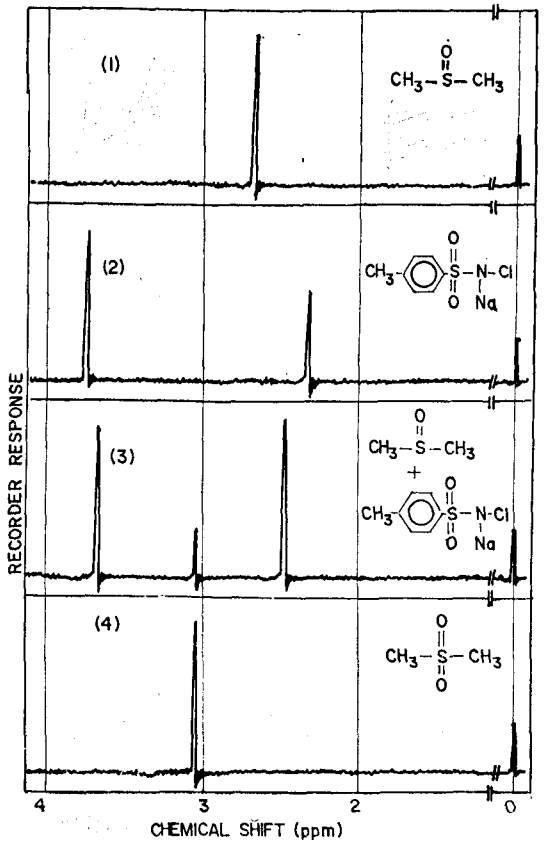

Fig. 3. A sketch of NMR spectra upto 4ppm region

It is known, and also has been confirmed that maintaining the bindability of the labelled insulin to its antibody and enhancing the labelling yield is generally contradictory to each other[14]. Further it is considered that the bindability of the labelled insulin to its antibody is anyway more important than enhancing the labelling yield. Thus, in so far as the bindability is concerned, obtaining the highest labelling yield in the extent of maintaining the bindability of the labelled insulin is important. When DMSO was added as a labelling-aid, the amount of the DMSO was fixed to 10 ul which was proposed to be adequate enough for radioiodine labelling of gastrin[8].

The labelling yield was degenerated in

definitely short reaction time comparing those obtained without DMSO. (Table 1) (Fig. 1). As Table 1 shows, in absence of DMSO, the reaction should be terminated in 3 sec obtaining the labelling yield of about 10%. It has been confirmed that the labelling yield could not be increased even if the reaction time was extended without the labelling-aid. In the presence of DMSO, however, by extending the reaction time the yield could be increased up to 30% in 60 sec. The cause of such a trend is attributable to the retardation of the reaction rate by DMSO since it plays a role of mildening the reaction conditions; the oxidizing power of the chloramine-T may be dispersed in so far as the DMSO is oxidized as well as the sulfide linkage in the peptide hormone. Thus, when the labelling-aid was applied, the reaction needed not to be stopped in a hurry to prevent the denaturation of the labelled product as previously[14]. The increased yield is consistent with those in the literatures[8]. The specific activity of the labelled product roughly estimated from the yield was about 100 uCi/μg.

3.2. The Bindability of the Labelled Insulin to the Antibody

As Fig. 1 shows, the bindability was remarkably increased when the labelling-aid use was consisting with those in the literatures[8, 10]. Thus, DMSO is considered to be useful both for enhancing yields and increasing bindabilities. The cause of such a fact may be attributable to the oxidation of DMSO (see below) and the consequent diminiation of the oxidation of sulfide in the peptide hormone to maintain its biological and immunological activity. As Table 1 shows, the average B/F value was increased from 1.5 to 2.5 in the definite antibody dilutions. Thus, the net increase of the bindability was 70%. The result is just consistent with those obtained for gastrin[8]. Further, as shown in the last column in Table 1, the value of B/F $\times$ dilution radio to yield 2×10^5 cpm/ml for each well labelled fraction of the labelling batch was increased from 55.63 to 116.85 by applying DMSO as a labelling-aid. As it is twice of the value obtained without DMSO, the productivity of the insulin radioimmunoassay kits is increased from 2 to 4.

As Fig. 2 shows, the B/F value was ranged from 0.17 to 1.20 in the standard dose-response curve plotted by using the labelled insulin obtained in the labelling-aid method, while it was from 0.17 to 0.90 in the ordinary method. It means that the labelled insulin obtained by the modified method has not lost much of the immunological activity. Since the steeper slope of the standard dose-response curve is quite important especially in the range of lower doses of standard in the assay, the merit of the labelling-aid would be estimated to be enormous for the production of the radioimmunoassay kits.

3.3. Identification of the Oxidation Product of DMSO

The characteristic peak originating from the six methyl hydrogens appeared at 2.7 ppm and 3.1 ppm, for DMSO and dimethylsulfone, respectively. (Fig. 3). In the preparation of NMR samples, the water soluble DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) could not be used as a standard since it is a sulfonate. Thus, a small amount of dioxane, (dioxane plus TMS in $CDCl_3$) was added as a standard. It has been confirmed that the peaks were shifted about 2 ppm to down field in the case of using

TMS (or DSS) as a reference standard. However, since the degree of shifts was nearly same for DMSO and dimethylsulfone, there was no problem in distinctions.

The chloramine-T showed its methyl hydrogens peak at 3.5 ppm. When DMSO was mixed with chloramine-T it was shifted to 4.5 ppm probably due to the methyl hydrogens of DMSO, and a new small peak was appeared at 3.1 ppm ((3) in Fig. 3) which was just consistent with the peak originating from the hydrogens in dimethylsulfone ((4) in Fig. 3). The peaks at 3.7 and 3.6 ppm in the (2) and (3) of the Fig. 3 were originated from the hydrogens in dioxane.

For further consolidation of the assignment of the peak at 3.1 ppm, a series of experiments was carried out varying the reactants concentrations and reaction temperatures. The concentration of chloramine-T, and DMSO (10 μl) in the labelling mixture is 0.1%, and 35%, respectively. Even though the peak at 3.1 ppm could not be found in the original labelling mixture, it was appeared when the concentrations of the reactants were increased to more than 10%, and the reaction mixture was kept at 25~28°C for more than 10 min. Thus, it has been identified that the DMSO is converted to dimethyl sulfone by chloramine-T in the labelling mixture. It is considered that the oxidation proceeds as the following reaction scheme;

$$\underset{\text{(chloramine-T)}}{CH_3C_6H_4NClNa}+H_2O\rightleftharpoons\underset{\text{(a sulfonamide)}}{CH_3C_6H_4SO_2NH_2}+NaOCl \quad (5)$$

$$NaOCl+H_2O\rightleftharpoons NaOH+HOCl \rightleftharpoons NaCl+H_2O_2 \quad (6)$$

$$\underset{\text{(DMSO)}}{CH_3SOCH_3}+H_2O_2\dashrightarrow\underset{\text{(dimethylsulfone)}}{CH_3SO_2CH_3}+H_2O \quad (7)$$

4. Conclusions

4.1. The DMSO in the labelling mixture of insulin played a role of a labelling-aid increasing the B/F ratio from 1.5 to 2.5 (70 % increase).

4.2. Even though the DMSO in the labelling mixture of insulin retarded the rate of the radioiodination, the average labelling yield was raised upto 30% by extending the reaction time to about 1 min.

4.3. Owing to the increased B/F ratios the productivity of the insulin radioimmunoassay kits was doubly increased. The dose gradients were also larger in the case of using labelled insulin obtained in the DMSO labelling-aid method than those obtained using the ordinary method.

4.4. It has been identified by a NMR spectrometry that the DMSO was converted to dimethylsulfone in the labelling mixture by the oxidative action of chloramine-T.

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