Radiation Aggregates of Insulin

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During the irradiation of aqueous solution of insulin (pH 1.8) the decrease of original insulin molecules and the formation of radiation aggregates of insulin was studied in dependence on the concentration of irradiation solution, on the doses and conditions of irradiation (oxygenated and oxygen-free atmosphere, the presence of *t*-butanol, addition of [^{14}C]amino acids). From the results obtained and results published earlier it may be assumed that in irradiated solution on insulin new covalent bonds between insulin molecules are formed at tyrosyl, phenylalanyl and cystyl radicals; these radicals are formed by the action of both direct and indirect effect (at present pH 1.8 mainly of H atoms).

The character of the bonds that are involved during the radiation aggregation of proteins is not yet quite clear. From the results reviewed in this field [1] it follows that in addition to hydrophobic and electrostatic interactions covalent bonds are also involved in radiation aggregation of proteins. Yamamoto [2–5] irradiated aqueous solutions of serum albumin in the presence of selected [^{14}C]amino acids and followed the yields of the radioactivity incorporated in the protein. Aromatic and sulphur-containing amino acids were found most reactive, indicating that the covalent bonds in radiation aggregates are formed predominantly between the radicals formed on Cys, Met, Try, Phe and Tyr residues of the irradiated protein molecule. Similar results were also obtained by Jakubick and Delincée [6] during the irradiation of an aqueous solution of myoglobin. From their results it follows that the radiation-induced binding of amino acids takes place at specific reactive sites of the protein.

Similarly as during the irradiation of the proteins the irradiation of aqueous solutions of insulin (3.1×10^{-4} M, pH 1.8) is also accompanied by a loss of the original insulin-monomer and by the formation of high-molecular radiation aggregates [7].

In the present paper we endeavoured to characterize these aggregates and their formation in dependence on the conditions of irradiation, on the presence of radical scavengers and various [^{14}C]amino acids. In contrast to the paper by Yamamoto we not only measured the total fraction of the incorporated radioactivity, but also the distribution into individual insulin fractions after its separation on a Sephadex G 75 column, and we also compared the effect of individual amino acids on the formation of radiation aggregates of insulin.

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Experimental Conditions

For the experiments solutions of insulin (from the firm NOVO) in redistilled water acidified with sulphuric acid (analytical grade) to pH 1.8 were prepared. The concentration of the solutions was in the range $0.775 - 3.1 \times 10^{-4}$ M. The molecular weight of the insulin monomer 5 730 was taken as the base of calculation of the molarity.

As radiation source ^{60}Co was used in a dose of 16.6 rads/sec intensity. The radiation does were in the 10 – 180 krads range. The samples were irradiated in glass ampoules. Some part of them was previously bubbled through with air while the rest was treated in the same manner with nitrogen and N_2O. Some ampoules were irradiated in the presence of *t*-butanol (0.2 M) while the other part was irradiated under bubbling through with oxygen.

In some experiments the following [U-^{14}C]amino acids were used: lysine, leucine, phenylalanine, tyrosine (Institute for Research, Production and Utilization of Radioisotopes, Prague) and cystine-[3.3′-^{14}C] (V/O Izotop, USSR). These radioactive amino acids, mixed with corresponding inactive standards (Fluka), were added to the solutions of insulin to give 10^{-3} M concentrations.

The irradiated and the non-irradiated solutions were separated chromatographically on a Sephadex G 75 column 76 cm high and 1.6 cm diameter with

Table I. The values of D_{37} and G_i (−M) found in irradiated solutions of insulin (in concentration range $0.775-3.1 \times 10^{-4}$ M) in oxygen-free and oxygen-limited atmosphere. (The solutions of insulin were bubbled through with nitrogen and with air before irradiation.)

Insulin concentration [$\times 10^{-4}$ M]		D_{37} [krads]	G_i (−M)
0.775	N_2	80	0.93
	air	186	0.41
1.55	N_2	88	1.72
	air	192	0.78
3.1	N_2	97.7	3.10
	air	210	1.43

Table II. Amino acid composition of radiation aggregates of insulin.

Amino acid	Theoretical yields	Standard sample of insulin	Radiation aggregates of insulin formed during the irradiation with the dose:	
			126.6 krads	180 krads
Lysine	1	1.0	1.0	1.0
Histidine	2	2.0	1.85	1.8
Arginine	1	1.1	1.1	1.1
Aspartic acid	3	3.0	3.0	3.1
Threonine	1	1.0	0.9	0.8
Serine	3	2.7	2.8	2.8
Glutamic acid	7	6.9	7.0	7.4
Proline	1	1.0	1.0	1.0
Glycine	4	4.1	4.0	4.0
Alanine	3	3.0	3.1	3.1
1/2 cystine	6	5.8	4.4	3.7
Valine	5	4.5	4.5	4.5
Isoleucine	1	0.6	0.6	0.6
Leucine	6	6.0	6.0	6.0
Tyrosine	4	3.6	3.4	3.3
Phenylalanine	3	2.9	2.9	2.8
Cysteic acid	0	0.0	0.8	1.2

The analyses wer standardized on leucine.

2.5 M acetic acid. The fractions of the effluents were measured on a Specord UV-VIS spectrophotometer at 276 nm.

The disappearance of the original molecules of insulin-monomer in the irradiated solutions and the formation of the radiation fractions of insulin were evaluated by comparison with the insulin peak eluted from non-irradiated solution.

For the study of radiation aggregates of insulin individual fractions from the Sephadex G 75 column were estimated by comparing the rate of their elution through the Sephadex G 75 column and also after freeze-drying, on plates made of Sephadex G100 and 150 (Superfine) with the elution rates of the standards: cytochrome C, ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin, and bovine serum gammaglobulin (all the samples were from Koch-Light Laboratories LtD.).

The formation of -SH groups in irradiated solutions of insulin was evaluated using Ellmans reagent [8].

The amino acid composition in insulin aggregates after their acid hydrolysis was determined with an amino acid analyzer (Developmental Workshops of the Czechoslovak Academy of Sciences, Prague). The estimation of the biological activity of these products, tested on rabbits, was described in a separate paper by Kopoldová and Nobilis [9].

The radioactivity of the effluents was measured using a liquid scintillation spectrometer (Isocap 300).

Results and Discussion

1. Decrease of insulin-monomer in dependence on the concentration and the dose of irradiation; formation of radiation aggregates

From the dependence of the decay of original insulin molecules content on the radiation dose the D_{37} values were evaluated and initial G_i $(-M)$ values were calculated. The results are given in Table I. It is evident that the initial yields G_i in an oxygen-free atmosphere are approximately double in comparison with the G_i yields found in an oxygen-limited atmosphere. In both cases the initial yields increase in proportion to the initial concentration of irradiated solution. (Limited solubility of insulin made further investigation in this line impossible.) Results in Table I correspond to the results obtained during the polarographic analysis of -S-S groups in X-irradiated solutions of insulin [10] and chymotrypsinogen [11]. According to these results it was assumed that both the direct and indirect radiation effect plays a role in radiolysis of aqueous solutions of some proteins and peptides. In the X-radiolysis of 3.6×10^{-4} M aqueous solution of insulin the direct effect plays the prevailing role ($G_i - 2.12$) whereas the role of indirect effect is minor ($G_i - 0.45$) [7].

Simultaneously with the decrease of insulin-monomer content the increase of insulin aggregates

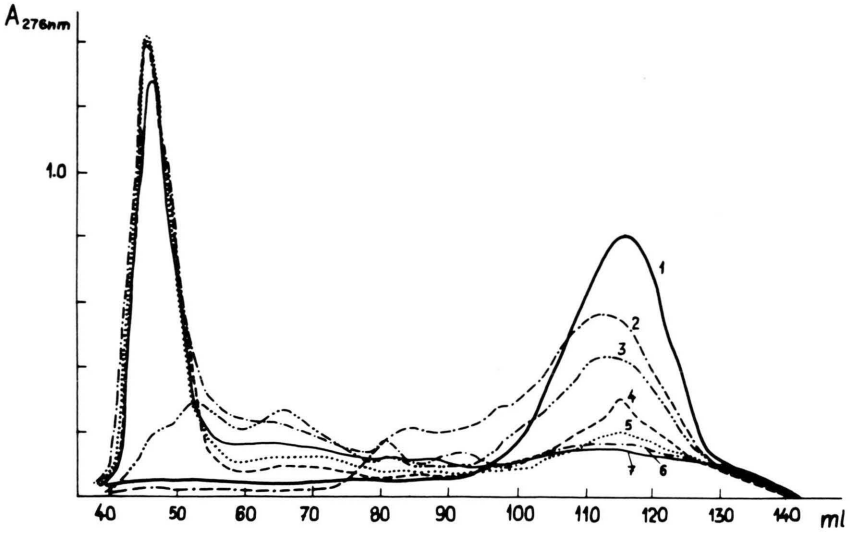

Fig. 1. Gel filtration of non-irradiated [1] and under various conditions irradiated [2 – 7] solutions of insulin (concentration 3.1×10^{-4} M, absorbed dose 180 krads) on the Sephadex G 75 column with 2.5 M acetic acid. 2-oxygen-saturated (– – · – –), 3-oxygen-limited (– · · –), 4-N_2O + *tert*-BuOH (– – – –), 5-N_2 + *tert*-BuOH (· · · · ·), 6-N_2O (– · –), 7-N_2 atmosphere (solid line).

was observed. Their formation was negligible in very diluted solutions but increased in dependence on the concentration of irradiated solution of insulin.

Irradiated 3.1×10^{-4} M aqueous solution of insulin (pH 1.8) was separated on the Sephadex G 75 column; the concentration decrease of the original insulin-monomer and the formation of higher molecular weight fractions were studied (Fig. 1). The eluates with a maximum at 52, 67 and 82 ml correspond to a molecular weight of about 36 000, 24 000 and 12 000, as estimated by comparison of their flow-rate through the column with that of the standard samples of cytochrome C, ribonuclease A and chymotrypsinogen A. The first fraction of eluates with the maximum at 47 ml corresponds to higher radiation aggregates. The molecular weights of these freeze-dried fractions correspond to 42 000, 60 000, 84 000, 120 000 and 160 000 according to their flow-rate through the thin-layer plates of Sephadex G 100 and 150 (superfine).

The formation and the size of radiation aggregates of insulin increases with increasing radiation dose. These results are presented in Fig. 2. The formation of lower-molecular weight fractions (about 24 000) has an exponential course, in the case of fractions with m. w. about 12 000 even a slight decrease was observed during the irradiation. In contrast to this the formation of high molecular aggregates (with m. w. up to 42 000 and higher) that takes place only very slowly at the beginning, increases in the course of irradiation. It may look like a series of consecutive pseudo-first order monomolecular reactions.

Amino acid analysis of radiation aggregates of insulin was carried out parallelly with a standard sample of non-irradiates insulin. The results are given in Table II. As is evident from the Table a decrease in the content of histidine, threonine, tyrosine and phenylalanine in addition to a mild increase in glutamic and aspartic acid content was found. A striking decrease was found in the content of cystine. After irradiation with 126.6 krads the number of cys/2 residues decreased by 25%, on irradiation with 180 krads up to 36%. It was shown by simultaneous determina-

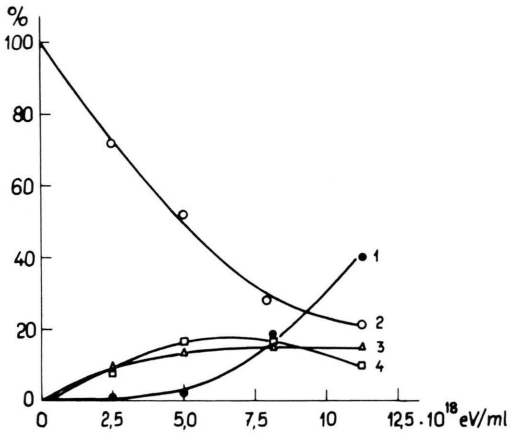

Fig. 2. Dependence of radiation decrease of original molecules of insulin irradiated under nitrogen in 3.1×10^{-4} M aqueous solution (2) and formation of radiation aggregates on the dose of irradiation. (1 – high-molecular weight aggregates, 3 – products with m. w. about 24 000, 4 – products with m. w. about 12 000.) Maximal dose of irradiation used in this experiment corresponds to 180 krads.

tion of cysteic acid that more than half of this decrease is caused by oxidation of sulphur atoms. In irradiated solutions of insulin the presence of -SH groups was also detected (about 1.5×10^{-4} M) indicating that in addition to oxidative reactions reductive processes on the sulphur atoms also take place.

From the results of bioassays [9] it followed that the biological activity of radiation aggregates of insulin formed after irradiation with 180 krads is about 38% lower than the activity of the same amount of non-irradiated insulin preparation, treated in the same manner. It seems that the decrease of the biological activity of radiation aggregates of insulin is mainly due to a radiation damage to the -S-S-groups (36%), and to a negligible extent also to the damage to some further amino acids.

2. The influence of the atmosphere and the presence of OH radical scavenger on the formation of radiation aggregates of insulin

Table III expresses the decrease of concentration of the original insulin-monomer and the formation of radiation fractions during the irradiation in the presence of oxygen, nitrogen and in the presence of *tert*-butanol. The presence of oxygen evidently prevents the aggregation process. However, partial denaturation of original molecules of insulin takes place, as is evident from the turbidity observed in solutions which were bubbled through with oxygen in the course of irradiation. During the separation of these solutions on a Sephadex column (Fig. 1) the proportion of the fraction 80 – 95 ml increases correspondingly, probably owing to the denaturated molecules of insulin.

In oxygen-limited atmosphere (solutions of insulin were bubbled through with air before irradiation) some radiation aggregates were formed, but to a limited extent; the fractions with lower molecular weights predominated.

In oxygen-free solutions the amount of high-molecular weight aggregates increased. In these cases their content attained almost 40% of the initial weight of insulin.

As evident from Table III, only small differences between the decrease of insulin-monomer concentration and yields of radiation aggregates were observed in solutions irradiated under nitrogen and in an atmosphere of N_2 + *t*-butanol. This indicates that OH radicals do not significantly affect the course of the aggregation by irradiation of insulin solution. At present pH (1.8) therefore, the only reactive radicals in radiolysis of aqueous solution of insulin are H atoms.

Table III contains some results of spectrophotometric evaluation of irradiated solutions of insulin where increasing absorbance at 276 nm was found. The increase was especially pronounced in isolated fractions of radiation aggregates. These results indicate that molecules of insulin, the secondary structure of which was affected during irradiation, take part in the formation of radiation aggregates, or the process of radiation aggregation itself causes some structural changes in insulin molecules.

3. Effect of added amino acids on the course of radiation aggregation of insulin

3.1×10^{-4} M aqueous solutions of insulin (pH 1.8) were irradiated under nitrogen in the presence of selected [^{14}C]amino acids. The results are summarized in Table IV. The total fraction of the incorporated radioactivity increases gradually from aliphatic amino acids, where it is about 1%, to aromatic amino acids (2 – 2.75%), up to sulphur-containing amino acid, where it attains 4.3%.

Table III. Decrease of the original insulin-monomer and the formation of radiation aggregates in a 3.1×10^{-4} M solution of insulin (pH 1.8) irradiated with a 180 krads dose. (The area of the peak of non-irradiated insulin was taken as 100%.)

Medium	Fraction 40 – 59 ml max 47 ml	max 52 ml	Fraction 60 – 75 ml max 67 ml	Fraction 76 – 85 ml max 82 ml	Fraction 86 – 95 ml max 90 ml	Fraction 96 – 130 ml max 115 ml insulin-monomer	Increase in absorption of total irradiated solution of insulin (in % of absorption of non-irradiated sample)
N_2	37		12	5	4	22	19
N_2 + *t*-BuOH	38		9	4	3	24	20
oxygen-limited	6	10	17	5	4	50	18
O_2	–	–	–	22		72	not measured (slightly turbid solution)

Table IV. Yields of radiation-induced binding in the fractions of insulin after irradiation of a 3.1×10^{-4} M aqueous solution (pH 1.8) under nitrogen with a 180 krads dose in the presence of 10^{-3} M ^{14}C-labelled amino acids (expressed in % of total radioactivity eluted from the Sephadex G 75 column).

[^{14}C]amino acid	Fraction 40–59 ml	Fraction 60–75 ml	Fraction 76–95 ml	Fraction 96–130 ml insulin-monomer	Total binding yield
Lysine	0.4	0.2	0.15	0.25	1.0
Leucine	0.4	0.15	0.15	0.28	0.98
Phenylalanine	0.05	0.15	0.4	1.3	1.9
Tyrosine	–	–	0.85	1.9	2.75
Cystine	–	–	1.3	3.0	4.3

The distribution of the incorporated radioactivity and the total elution profile of insulin irradiated in the presence of individual amino acids is interesting. The elution profile of insulin irradiated in the presence of lysine and leucine, *i. e.* of aliphatic acids, is in no way distinctly affected in comparison with insulin irradiated with the same dose but in the absence of amino acids. A somewhat higher concentration of the insulin-monomer (32 and 34% as opposed to 22%) and a negligibly lower content of high-molecular radiation aggregates (33 and 35%, as opposed to 37%) was found. When insulin was irradiated in the presence of aromatic amino acids and cystine the situation is different. In the presence of phenylalanine the content of the insulin-monomer decreased to 45% only and the radiation aggregation took place to a limited extent, with fractions with lower molecular weight prevailing. This trend is still more pronounced in the case of tyrosine (the content of the monomer decreased to 55%) and with cystine when the concentration of the insulin-monomer decreased to 70% only and the formation of radiation aggregates was completely limited. From the results mentioned it follows that the presence of aliphatic amino acids in the irradiated solution of insulin does not limit the formation of radiation aggregates; the incorporated radioactivity is distributed over all radiation fractions of insulin. The presence of aromatic and sulphur-containing amino acids, however, does limit the formation of radiation aggregates; the predominant part of the incorporated activity was found in the fraction of insulin-monomer where it is bound both covalently and physically. This is proved by the results which were obtained on hydrolysis of insulin-monomer isolated from the solution of insulin irradiated in the presence of [^{14}C]tyrosine. The hydrolysate of this fraction (6 N HCl, 106 °C, 16 h) gives on paper chromatography in *n*-butanol-acetic acid-water mixture (4 : 1 : 5) three maxima of radioactivity. About 13% corresponds to tyrosine which is evidently bound physically in the course of irradiation onto the insulin-monomer molecules. The main part of radioactivity remains on the start (30%) and near the start (about 25%) which according to its position corresponds to radiation recombinates formed on irradiation of aromatic amino acids [4, 5] and their mixtures with cysteine [2].

During the irradiation of insulin in the presence of [^{14}C]tyrosine or also other reactive amino acids covalent binding between the protein and the added amino acid takes place preferentially, while the formation of radiation aggregates is limited. The totally low extent of radioactivity incorporated into insulin fractions indicates that the formation of covalent bonds evidently takes place at specific sites only.

On the basis of present results and results obtained in previous papers [7, 10, 11] it may be assumed that in the formation of new covalent bonds between insulin molecules the radicals at tyrosyl, phenylalanyl and cystyl residues are involved which are formed by the action of both the direct and indirect effects (at pH 1.8 mainly of H atoms).

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