RADIOLYSIS OF AQUEOUS SOLUTIONS OF INSULIN

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Received 5th July, 1974

The effect of ionizing radiation in diluted aqueous solutions of insulin is analyzed. The rate of its decrease (the loss of polarographic activity) is described by a first-order kinetic equation. The results lead to a concept of a direct influence on macromolecules in "excited volumes". The amount of inactivated molecules (*i.e.* the yield) within this volume is proportional to the solute concentration. In diluted aqueous solutions also an indirect radiation effect takes place. The paper concerns an evaluation of this effect.

In our previous studies the concentration of insulin was followed by measuring the polarographic double-wave[1-3] of the disulphidic groups of the insulin molecule[4,5]. The concentration measured in this way is proportional to the concentration of polarographically "active" disulphidic groups. In this way, a direct oxidation or an inactivation of the disulphidic group cannot be distinguished from an occasional loss of the polarographic activity in some other way, *e.g.*, as a result of a change in the secondary or tertiary structure of the molecule, or of the formation of a dimer, trimer, or a like aggregate, where "masking" of the polarographic activity takes place. By the total sample hydrolysis peptides may be split into free amino acids. Of those, cystin exhibits the polarographic activity in the same way as insulin.

In this communication the influence of the "masking" effect was excluded by a polarographic study of the hydrolysate or irradiated samples. Also, information was obtained on the indirect inactivation of disulphidic bonds. A considerable part of this communication deals with the confirmation of the hypothesis concerning the formation of an insulin subunit aggregates.

EXPERIMENTAL

An insulin sample (Lachema) commonly used for medical purposes was used in this study; it contained 0·4% Zn (1 mg of the original sample corresponds to 23·7 biological units). Stock solutions were prepared by dissolving a calculated amount of the sample in 50 ml of triply-distilled water whose pH was adjusted to 1·6—1·8 by adding 96% sulphuric acid of analytical purity. The calculation of insulin molarity was carried out using its molecular weight (5733 for one subunit)[6]. The insulin concentrations in the irradiated samples were: $3{\cdot}50 \cdot 10^{-4}$M; $1{\cdot}75 \cdot 10^{-4}$M; $0{\cdot}87 \cdot 10^{-4}$M; $0{\cdot}44 \cdot 10^{-4}$M. The samples were irradiated by X-rays using a Micrometra II appa-

ratus (voltage 50 kV, a MACHLETT AEG 50 X-ray tube with a tungsten cathode and a beryllium window). The dose intensity was determined by the ferrosulphate dosimeter of $G_{Fe^{3+}} = 13{\cdot}1$ (ref.[7]). The air-saturated samples (6 ml) were irradiated in Petri dishes of 4·5 cm diameter ($I = 3{\cdot}9 \cdot 10^{16}$ eV ml^{-1} s^{-1}). During the irradiation the samples were agitated by a magnetic stirrer. After the irradiation the samples were subjected to hydrolysis: to 5 ml of the solution in a 50 ml flask 4·5 ml of 37% hydrochloric acid (analytical purity) was added, the mixture was heated to the boiling point under a reflux in a sand bath for 4 hours. After the complete hydrolysis was achieved, the sample was neutralized by 25% ammonium hydroxide of analytical purity (about 4·2 ml) up to pH about 7, and distilled water was added up to 100 ml. About 0·2 to 0·5 ml of such a sample was taken and mixed with 9·8 to 9·5 ml, respectively, of the testing cobalt solution ($2 \cdot 10^{-3}$M-$CoCl_2$, 0·1M-NH_4Cl, 0·1M-NH_3). Polarographic curves were recorded in the potential region of the cystine catalytic wave. The concentration of the remaining cystine after the hydrolysis of the irradiated samples of insulin was obtained from the calibration curve, determined with a non-irradiated hydrolyzed insulin sample treated in the same way. To determine the molecular weight of radiation products, $2{\cdot}1 \cdot 10^{-4}$M insulin solution was used, prepared in the way mentioned above. The samples were irradiated by various doses of X-radiation ($I = 2{\cdot}4 \cdot 10^{16}$ eV ml^{-1} s^{-1}) and — without being hydrolyzed — they were subjected to separation on a Sephadex G75 column using 2·5N acetic acid for elution. The product molecular weight was estimated both from the elution volumes during the separation on the Sephadex G75 column and from the results of thin-layer chromatography (Sephadex G100 and G150-superfine) using a comparison with samples of standard proteins. By comparing the elution volumes of the standard proteins samples with those of the elution radiation products, molecular weights of these substances were calculated. The relative evaluation of radiation products of the insulin subunit aggregation was made spectrophotometrically (Fig. 1).

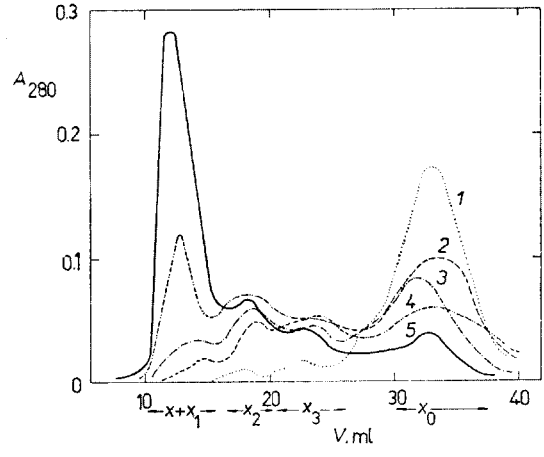

FIG. 1

Separation of Irradiated Insulin Samples on Sephadex 75 Column

Absorbed dose: *1* not irradiated sample; *2* 5·76; *3* 8·89; *4* 11·52; *5* 14·4 . 10^{18} eV/ml. .

RESULTS AND DISCUSSION

Experimental results obtained by studying the insulin concentration decrease (following the height of the cystine catalytic wave after insulin sample hydrolysis) in dependence on the irradiation dose shows that this decrease follows the first-order equation. By plotting the logarithm of the relative concentration of the solute S/S_0 (S_0 is the initial, S the instantaneous concentration) against the absorbed dose $D(It)$ at the same dose rate I ($3{\cdot}9 \,.\, 10^{16}$ eV $ml^{-1}s^{-1}$) a series of linear dependences for various initial solute concentrations obtains (Fig. 2). The insulin decrease measured polarographically (after the insulin sample hydrolysis) can be in general described by the first-order kinetic equation

$$S = S_0 \, e^{-kD} , \qquad (1)$$

where k are the corresponding rate constants as determined by the slopes of the straight lines.

It was found experimentally that the dose D_{37} (*i.e.* the dose necessary to decrease the relative concentration S/S_0 to $1/e = 0{\cdot}37$) is dependent on the initial concentration of the solute S_0. According to (*1*) it holds for D_{37}

$$kD_{37} = 1 . \qquad (2)$$

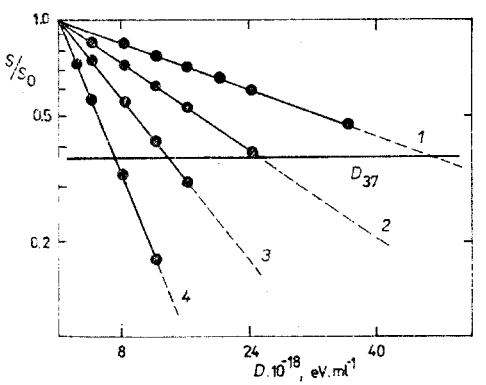

FIG. 2

Dependence of Relative Decrease of Insulin Concentration S/S_0 after Hydrolysis on Absorbed Dose for Various Initial Concentrations of Insulin S_0

10^{-4}M: *1* 3·50; *2* 1·75; *3* 0·87; *4* 0·44.

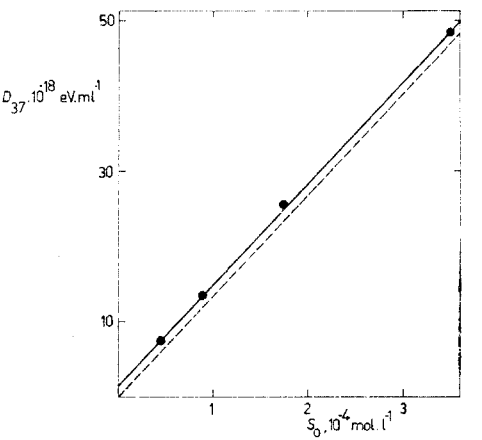

FIG. 3

Dependence of the Experimental Value D_{37} (full line) and the Corrected Value D'_{37} with Substance L Absent (dashed line) on the Initial Concentration of Insulin S_0

The values of D_{37} derived from Fig. 2 are linearly dependent on S_0 (Fig. 3) and can be written as

$$D_{37} = \frac{S_0 + C}{A} . \tag{3}$$

It can be seen from equation (2) and from the experimentally found relation (3) that the value of the rate constant k decreases with the increasing concentration of the solute S_0 according to the relation

$$k = \frac{A}{S_0 + C} , \tag{4}$$

where A and C are experimental constants. The decrease of the insulin concentration with the increasing dose $It = D$ thus obeys equation (1). By substituting (4) into the differentiated form of equation (1), $-\mathrm{d}S = kS\,\mathrm{d}D$, the experimental data can be described by the equation

$$-\frac{\mathrm{d}S}{\mathrm{d}D} = \frac{AS}{S_0 + C} . \tag{5}$$

With regard to equation (1) the radiation yield G (*i.e.* the number of inactivated molecules of cystine on 100 eV of energy absorbed in 1 ml) is given by[2,3]

$$G = \frac{6{\cdot}023 \,.\, 10^{22}(S_0 - S)}{D} = \frac{6{\cdot}023 \,.\, 10^{22} S_0 (1 - \mathrm{e}^{-kD})}{D} . \tag{6}$$

By extrapolating the G value to zero absorbed dose, $D \to 0$, the expression for the initial yield G_i is

$$G_i = 6{\cdot}023 \,.\, 10^{22} k S_0 = 6{\cdot}023 \,.\, 10^{22} \frac{AS_0}{S_0 + C} . \tag{7}$$

If $S_0 \gg C$, then

$$G_i = 6{\cdot}023 \,.\, 10^{22} A . \tag{8}$$

The independence of G_i of the solute initial concentration S_0 has been explained in many studies as a result of an indirect radiation effect on solute molecules[8-11]. We will attempt to explain this case more in detail using a simple model.

Let k_1 represent the rate constant of the (desactivation) reaction of solute molecules with a radical R, k_2 the rate constant of the reaction of these radicals with the inactivated solute molecules, and k_3 the rate constant of the reaction of the radical R

with a substance L present in the solution. The probability P that the radical inactivates the solute molecules is then given by

$$P = \frac{k_1 S}{k_1 S + k_2(S_0 - S) + k_3 L} . \qquad (9)$$

S denotes here the number of solute molecules in 1 ml, $(S_0 - S)$ the number of inactivated molecules in 1 ml, and L the number of molecules of another substance (scavenger) in 1 ml.

The initial yield G_i of the solute decrease caused by the radical reactions will be equal to the radical yield G_R multiplied by the probability P. Therefore, it holds

$$-10^2 \left(\frac{dS}{dD}\right) = G_R P . \qquad (10)$$

As the reactions of radicals with insulin or cystine and its inactivated form are diffusion-controlled processes, it may be assumed that k_1 is approximately equal to k_2 and then

$$-10^2 \left(\frac{dS}{dD}\right) = G_R \frac{S}{S_0 + (k_3/k_1) L} . \qquad (11)$$

In equation (*11*) for $D \to 0$ (*i.e.* $t \to 0$) it holds that $S = S_0$, and the equation can be compared with equation (*7*):

$$-10^2 \left(\frac{dS}{dD}\right)_{t \to 0} = G_i = G_R \frac{S_0}{k_3 L/k_1 + S_0} = 6{\cdot}023 \,.\, 10^{22} \frac{A S_0}{S_0 + C} . \qquad (12)$$

It follows then from (*12*)

$$6{\cdot}023 \,.\, 10^{22} A = G_R \text{ and } \frac{k_3 L}{k_1} = C . \qquad (13)$$

These considerations have been confirmed experimentally, as can be seen from Table I and Fig. 3. Therefore, it can be said that over the studied range of radiolysis an indirect radiation effect plays a role. (For C/A the value $1{\cdot}5 \,.\, 10^{18}$ eV/ml was found graphically from Fig. 3.) The G_i value determines the number of inactivated insulin molecules on 100 eV of absorbed energy *via* the indirect effect, the substance L acts as a scavenger.

If $L \ll S_0$ or if the substance L is absent, equation (*12*) will be identical with

equation (*8*) (*i.e.* $G_i = G_R$). Equation (*3*) reads under these conditions

$$D'_{37} = \frac{S_0}{A} . \qquad (14)$$

The dependence is shown in Fig. 3 (dashed) and the observed values of D'_{37}, k', and G'_i which fit this straight line are given in Table I. From equation (*14*) the value $A = 7{\cdot}4 \,.\, 10^{24}$ mmol/eV can be calculated. Substituting this value of A into equation (*3*), assuming $S_0 \to 0$, the value $C = 1{\cdot}11 \,.\, 10^{-5}$ mmol/ml is obtained.

The results and considerations of this paper and the results obtained earlier[2,3] make it possible to summarize the information about the radiolysis of aqueous solutions of insulin in the following way: during the radiolysis of an aqueous solution of insulin both the direct and the indirect radiation effect plays a role. The insulin molecules in the aqueous solution are subjected to the primary attack within the excited volume[3]*) by the ionizing radiation (*via* a direct energy transfer from a spur to protein molecules or *via* a moderation of an electron which may migrate during thermalization in a spherical volume with radius up to 5–15 nm, though its total path is several times longer). This shows itself in the decrease of the concentration of the irradiated non-hydrolyzed sample (measured polarographically). This decrease obeys the first-order kinetics, where the rate constant is independent of the initial concentration and the radiation intensity.

Primarily affected insulin molecules (they loose their polarographic activity as a result of aggregation and "masking", *i.e.* as a result of a change of the secondary and tertiary structure) are indirectly attacked even in the following stages by the radicals formed in the excited volume or outside of it. The indirect radiation effect manifests itself here on the disulphidic bonds of cystine molecules or on other amino-acids.

The polarographic investigation of non-hydrolyzed irradiated insulin[2] and – after irradiation – of hydrolyzed insulin (this study) made it possible to estimate both the direct and the indirect effect. (The yield G_i from non-hydrolyzed insulin depends on the initial insulin concentration S_0 ($0{\cdot}45-3{\cdot}6 \,.\, 10^{-4}$ mol/l) within the range $G_i = 0{\cdot}3-2{\cdot}12$.)

If the concentration of insulin in the solution is small, and few or almost no insulin molecules occur within the excited volume, the direct effect plays no role. The insulin loss will be then a function of the indirect effect only.

Energetic changes connected with the changes of the secondary or tertiary structure are very small and may be explained by a loss of energy of the moderated electron

* The excited volume was calculated from the rate constant ($k = 10^{-19}$ eV ml^{-1}) of the unimolecular loss of the irradiated non-hydrolyzed insulin[2], similarly as in ref.[3]; the radius of this volume is 9·45 nm.

prior to its solvatation. Thus, it is possible to accept the notion of the "excited volume" as a volume in which the moderation of the electron takes place, as explained in the earlier paper[3].

The subject of this study was also the investigation of the aggregation of irradiated non-hydrolyzed insulin. It was necessary to verify the assumption that by the direct effect within the "excited volume" masking of the polarographic activity of insulin molecules results from the increasing molecular weight of insulin[3].

The changes which take place in an aqueous solution of nonhydrolyzed insulin irradiated by stepwise increasing doses can be seen in Fig. 1. In the irradiated solution radiation products of a higher molecular weight were formed. In the sample irradiated by the dose $D = 5{\cdot}8 \cdot 10^{18}$ eV ml^{-1} these products were the substances whose elution volumes V_e corresponded to 23·5 and 18·5 ml; they were denoted as products X_3 and X_2, respectively. When the irradiation dose was further increased, radiation products X_1 and X were formed of the elution volumes 12·5 and 12 ml, respectively. The maxima of the peaks measured shifted during the irradiation towards these substances. From the dependence of the elution volumes on the molecular weight of the standard proteins the molecular weights of the radiation products were estimated: for $X_3 \approx 18000$, for $X_2 \approx 25000$.

In view of the fact that the elution volumes of the products X_1 and X are too close together and merge one into the other, when the separation was carried out on Sephadex G75, other two types of Sephade, G100 and G150, were used. The migration distances were compared with those of the γ-globulin standard, and from there the molecular weight was estimated: $X_1 = 42000$, and $X = 60000$. It follows from the graphic plot that the decrease of insulin is accompanied by the formation of X_3, X_2, X_1, and X. These products are formed obviously as secondary radiation products by the aggregation of the products formed earlier.

TABLE I

Experimental Data (D_{37}, k, G_i) and Corrected Values (D'_{37}, k', G'_i) as a Function of the Initial Insulin Concentration S_0

$S_0 \cdot 10^4$ mol/l	D_{37}	D'_{37}	k	k'	G_i	G'_i
	10^{18} eV/ml		10^{-20} ml/eV			
3·50	48·5	47·00	2·06	2·15	0·43	0·45
1·75	25·4	23·50	3·94	4·25	0·41	0·45
0·87	13·4	11·75	7·47	8·50	0·39	0·45
0·43	7·2	5·85	13·90	17·1	0·36	0·45

So far the question of the bond among the molecular aggregates of radiation polymers has not been explained more in detail. In our preliminary experiments on the incubation of the isolated radiation products in 8M urea and in 10M guanidine it was found (on Sephadex) that no change of the molecular weight occurs. Therefore, it does not seem probable that hydrogen bonds would play a role in the radiation products. It may be assumed that intermolecular covalent bonds[12] prevail when radiation polymers are formed. A more detailed elucidation of this problem remains a subject for further studies.

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Translated by Z. Herman.