PREPARATION OF INSULIN DERIVATIVES DIFFERING IN THEIR BIOLOGICAL AND ANTIGENIC ACTIVITY

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UDC 615.363.379.012.6

The effect of chemical modifications of insulin on its biological and immunological activity is described. The hormone was iodinated, esterified with methanol, treated with formaldehyde, with performic acid, and by thermal denaturation, and was combined with diazonovocainamide to 50, 75, and 100% saturation. All the procedures listed above caused loss of the hypoglycemic activity, but the antigenic properties of the hormone were preserved completely after methylation and after coupling with diazonovocainamide to the extent of 75 and 50%.

The development of some pathological disturbances of carbohydrate metabolism can be linked with excessive secretion of insulin. Inhibition of insulin production or its neutralization in the blood can be achieved by immunization with active hormone. However, this principle is difficult to put into practice in the case of insulin because of its hypoglycemic activity, its low molecular weight, and its low immunogenicity, making large doses of the hormone necessary. To overcome these difficulties, insulin with its biological activity completely or partly blocked, yet retaining its original antigenicity, must be used, as has been successfully carried out with other hormonal preparations (the anahormones) [1-5].

In the investigation described below, various methods of treatment of the insulin molecule were studied with the object of obtaining a preparation deprived of biological activity yet retaining its antigenic properties.

EXPERIMENTAL METHOD

The following types of insulin were used: a) bovine and porcine insulin, in a solution with activity of 40 i.u./ml, after preliminary dialysis and lyophilization, or b) crystalline bovine insulin with activity of 20-22 i.u./mg. By contrast with the original methods of treatment, before iodination, methylation, and treatment with formaldehyde, the insulin was treated with the detergents nonylphenol and sodium dodecylsulfate, which were added to a 0.2% solution of the hormone in the corresponding medium in a final concentration of 0.1%. The subsequent treatment of the insulin and the oxidation with performic acid were carried out by the methods described previously [4, 10] with the exception of certain procedures which will be described below.

Azo-coupling with Novocainamide. The diazo compound from novocainamide was prepared before use by treating a solution of novocainamide hydrochloride with 1 M $NaNO_2$ in the presence of 1.5 M HCl at 0°C and a controlled minimal excess of HNO_2. The prepared diazo compound was diluted to a final concentration of 0.04 M. Insulin azoprotein was prepared by treating the insulin with the solution of the diazo compound: 100 mg of the hormone was dissolved in 1 ml 0.1 M NaOH solution, the solution was neutralized with 0.1 M HCl to pH 7.0-7.5, and the pH was adjusted to 8.5 by the addition of 1 ml 8% $NaHCO_3$ solution,

Laboratory of Endocrinology, N. N. Petrov Research Institute of Oncology, Ministry of Health of the USSR, Leningrad. Central Research Laboratory, University of Tartu. (Presented by Academician of the Academy of Medical Sciences of the USSR V. I. Ioffe.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 72, No. 8, pp. 75-78, August, 1971. Original article submitted December 1, 1970.

TABLE 1. Effect of Chemical Procedures on Biological and Antigenic Activity of Insulin

Procedure	Biological activity	Antigenic activity
	percent of original	
Control (original insulin)	100	100
Methanol–HCl*	2.6	100
Formaldehyde	0.1	0
Iodine (10-12 g-atoms/mole)	0	0
Diazo-coupling with novocainamide:		
coupling by 100%	0.1	50
" " 75%	0.4	100
" " 50%	0.8	100
Diazo–coupling with novocainamide and methylation	0.1	0
Performic acid	0	0
Thermal denaturation	0	0

*Incomplete replacement of the carboxyl groups was obtained during the methylation.

after which 0.05 ml samples of 0.01% solutions of sodium and p-nonylphenolpolyethoxyethanol dodecylsulfates were added. After 1 h the medium was made alkaline to pH 9.0 with Na_2CO_3 solution, and the diazo solution was added at 0°C until the diazo reaction had not disappeared for 15-20 min. The azoproteins were isolated after adjustment of the pH to the isoelectric point, evaporation in vacuo, and dilution with ethanol. The residue was separated by centrifugation.

Azo-coupling with Diazonovocainamide and Methylation. Insulin, coupled with diazonovocainamide to the extent of 50% saturation, was methylated by the method described above.

Thermal Denaturation. A sample of insulin weighing 100 mg was dissolved in 0.1 M NaOH solution and neutralized with 0.1 M HCl. The solution was kept in a boiling water bath for 30 min, and then dialyzed and lyophilized.

The hormonal activity of the insulin and its derivatives was determined by intravenous injection into rabbits, in which the blood sugar was determined after 30, 60, and 120 min by the orthotoluidine method [12].

Immunological activity was detected by the complement fixation test conducted at 4°C with three increasing doses of complement. The antisera used were prepared by immunizing guinea pigs with insulin, and they were exhausted with bovine and porcine serum proteins to rule out the possibility of nonspecific crossed reactions. The sensitivity of the method was 0.00025 mg insulin/ml. Crossed reactions with proteins of the species of animals from which the insulin was obtained were absent.

EXPERIMENTAL RESULTS

The results obtained by the use of different methods to inactivate the insulin are given in Table 1.

It is clear from Table 1 that insulin, after treatment by the various reagents, lost its biological activity and its specific antigenicity to a greater or lesser degree in all cases. The exception was azo-insulin. Azo-coupling with diazonovocainamide to 100, 75, and 50% saturation led to the almost total loss of biological activity, but immunological activity remained intact after coupling to 75 and 50% of complete saturation. After 100% coupling the antigenicity was reduced by 50%, and the greatest loss of biological activity was also observed. These results are interesting because in recent years considerable attention has been paid to the study of the active centers of insulin. Bromer et al. [8] and Arquilla et al. [7] consider that the N-terminal end of the B-chain and the N-terminal end of the A-chain and lysine in the B_{29} position are responsible for immunological activity. The same centers are responsible for biological activity, because treatment with fluorescein, which is added successively to the N-terminal ends of the A- and B-chains and to the lysine at B_{29}, leads to total loss of both biological and immunological activity. Arquilla et al. [6] have shown that I^{125} when incorporated into position A_{14} or A_{19} reduces both the antigenicity of the hormone and its hormonal effect. Grodsky et al. [11] subjected the insulin molecule to chemical action with dinitrobenzene sulfate, which reacts with the epsilon-amino groups of lysine, with azosulfonylic acid, reacting with tyrosine and histidine, with methanol, and by oxidation. Under these circumstances 6

of 12 or 11 of the 12 carboxyl groups were methylated. No loss of activity was observed after methylation of six carboxyl groups. Brunfeldt et al. [9] iodinated insulin with different numbers of iodine atoms per molecule (1-10 g-atom/mole) and found that loss of hormonal properties and antigenicity of the protein takes place progressively with an increase in the number of iodine atoms in the insulin molecule. Total loss of activities was observed after the incorporation of not less than 10 atoms per molecule. In recent years considerable attention has been paid to the manufacture of synthetic insulin and the investigation of its properties with an increasing number of added amino acids. Wilson [13] for instance, investigated individual segments of the insulin chains by their interaction with antibodies against bovine insulin and against the isolated A- and B-chain. Segment A_{10-16} reacted with anti-insulin serum just as well as the whole molecule, and, moreover, in a smaller dose. Most probably the hapten groups in this segment are the leucine residue at A_{13} or the tyrosine at A_{14}. In the D-chain, B_{1-8} and B_{24-30} are antigenically important. However, other work [6-8, 11] has shown that they are partly responsible for the hormonal activity of the protein also. These results thus apparently indicate a direct relationship between the biological and antigenic properties of insulin. However, the results of the present investigation for the coupling of insulin with diazonovocainamide, a substance known to be added to the phenolic and imidazole groups of protein, would seem to give greater support to the view that the active centers in the molecule associated with its biological functions and its antigenic properties are different.

The separation of the hormonal activity of insulin from its antigenicity by diazo-coupling allows antisera against the hormone to be obtained. Immunization with the derivative may be more successful than immunization with the hormone, not only because larger doses of the protein can be injected, but also because of the appearance of additional antigenicity, the presence of which can be postulated by analogy with other diazo compounds of the hormones [5].

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