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Biological Inactivation of Proteins by the Maillard Reaction. Effect of Mild Heat on the Tertiary Structure of Insulin

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An average of 3.7 hexose residues bound the reactive amino groups of insulin during 120 days of storage with D-[U-^{14}C]glucose at 37 °C and 70% relative humidity. Dimethylaminonaphthalenesulfonylation of insulin reacted for 15 days corroborated the preferential binding at the N-terminal residues (A_1-Gly and B_1-Phe). While the acid solubility increased tenfold at 15 days, suggesting dissociation of the hexamer into dimers, there was little or no change in its structural conformation as attested to by two biological functions. The 15-day Maillard insulin retained 78.2% of the ability to depress the level of blood glucose in rabbits, and 100% of its capacity to raise blood tryptophan in young rats. These results contrast with those for the reaction at 55 °C in which a large number of sugar residues have been demonstrated to bind the protein molecule during 1 month of storage.

In the Maillard reaction, reducing sugars form condensation products with the amino groups of amino acids, peptides, and proteins. At the initial stages, α-*N*-1-deoxy-2-ketohexose derivatives have been isolated and characterized from the reaction of glucose with amino acids (Heyns and Noack, 1964) and peptides (Heyns and Rolle, 1959). The Maillard reaction of proteins has been studied mostly on food proteins which appear to lose considerable solubility, digestibility, and biological value during the course of the reaction. In previous studies we found that egg albumin reacted under mild conditions (37 °C, 68–70% relative humidity) lost 50% of its nutritive value in the first 10 days, long before the appearance of any detectable discoloration (Tanaka et al., in press). We have suspected, therefore, that irreversible loss of nutritive value could occur in food proteins prior to the observation of extensive physicochemical changes.

Insulin is a well-defined small protein in which the early steps of the Maillard reaction could be studied. Schwartz and Lea (1952) determined that in the insulin–glucose system, the order of reactivity at 37 °C is B_1-phenylalanine > A_1-glycine > B_{29}-lysine but no mention was made of the stoichiometry of the reaction. In an effort to elucidate the structure of the brown pigment, Clark and Tannenbaum (1974) used insulin to demonstrate that up to 31 hexose residues could bind and cross-link the reactive amino groups of the polypeptide after browning for 37 days at 55 °C.

A study of the first physicochemical events which may bear on the biological properties of a Maillard protein must be made under reaction conditions that result in minimal polymerization and cross-linking at the reactive amino groups. The objective of this work was to modify crystalline insulin by the Maillard reaction under mild conditions (37 °C), measure the average number of hexose residues bound to the protein in the span of 4 months, and estimate the extent of early structural changes by observing the performance of the hormone in two different biochemical functions.

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EXPERIMENTAL SECTION

Browning of Insulin. Reaction mixtures were prepared in 20-ml glass vials by suspending 50 mg of crystalline insulin (bovine pancreas, 0.5% zinc, Sigma Chemical Co., St. Louis, Mo.) in 10 ml of 7.75% glucose, and lyophilizing to a final temperature lower than 10 °C. The vials were then stored uncapped in sealed chambers at 37 °C and 68% relative humidity for up to 4 months. For the glucose binding studies, each vial also contained from 20 to 50 μCi of D-[U-^{14}C]glucose (International Chemical and Nuclear Co., Cleveland, Ohio).

Glucose Binding. For every time point, the contents of a vial were dissolved in 10 ml of an acetic acid solution (approximately 5%, pH 2.6), and 0.3 ml was loaded onto a Bio-Gel P-6 (200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) column, and eluted with the same acetic acid solution. Other conditions for the gel filtration were: column size, 1 × 50 cm; flow rate, 22 ml/h; void volume, 18 ml; fraction volume, 1.5 ml. Fifty microliters of each fraction was counted in a liquid scintillation counter. The ninhydrin pattern was obtained after hydrolysis of 0.15 ml of each fraction with 1 ml of 2.5 N KOH at 121 °C for 40 min, followed by neutralization with 30% acetic acid. Unreacted glucose was determined in the fractions by the Glucostat method (Worthington Bio-Chemicals, Freehold, N.J.).

Glucose-free Maillard insulin samples (10–15 nmol) were dansylated (8-dimethylamino-1-naphthalenesulfonylated) and analyzed by the method of Gros and Labouesse (1969) using Sequanal grade urea (Pierce Chemicals, Rockford, Ill.). For this procedure the thin-layer chromatography (TLC) plates were spotted, developed, and scraped promptly to minimize the loss of fluorescence. The fluorescent material collected from the spots was quan-

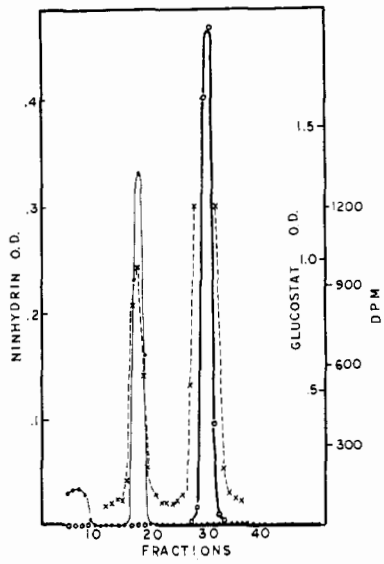

Figure 1. Bio-Gel P-6 column separation of unreacted and insulin-bound hexose. The first peak corresponds to normal and/or Maillard insulin. The second peak is unreacted glucose. A typical load consisted of 0.257 μmol of insulin and 6.177 μmol of originally D-[U-^{14}C]-glucose (sp act. 3.2 × 10^5 dpm/mg) in 0.3 ml of acetic acid (pH 2.6). The pattern was obtained by the glucose oxidase/peroxidase (Glucostat) reaction, by the alkaline hydrolysis/ninhydrin reaction, and by scintillation counting: ninhydrin (•–•); Glucostat (○–○); radioactivity (×- - -×).

titated in a Perkin-Elmer-Hitachi MPF-4A fluorescence spectrophotometer.

Biological Assays of Maillard Insulin. Batches ten times the size of those described above were stored for 15 days. The unreacted sugar was removed by washing with distilled water and centrifuging three times. This procedure provided 95–98% recovery of the insulin as monitored by the alkaline hydrolysis–ninhydrin test. The biological activity of the resulting insulin was determined by the blood-sugar rabbit assay described in the U.S. Pharmacopeia. This experiment was conducted on normal rabbits (Glochester Rabbitry, Glochester, R.I.) which were preconditioned to receive insulin injections in order to minimize extreme responses. On different days the rabbits were injected with the two kinds of insulin in such a way that each animal was used as its own control. The activity of the Maillard insulin was further tested in its ability to raise blood tryptophan levels in young rats. Three groups of eight rats (Charles River Breeding Laboratories, Wilmington, Mass.) each were assigned to receive injections (2 U/kg) of either normal insulin, Maillard insulin, or saline solution. The elevation of tryptophan in the plasma was monitored by the method of Fernstrom and Wurtman (1972).

The stability of the Maillard insulin in the presence of deacylases was tested in vitro. Fifteen-day Maillard insulin of high specific ^{14}C activity (180 mCi/mmol) was incubated with a rat kidney homogenate at 37 °C for 0, 1, 2, and 4 h according to Price and Greenstein (1948). The reaction was stopped with 1.5 vol of 10% trichloroacetic acid (Cl_3CCOOH), the precipitate was sedimented, and the radioactivity of the supernatant was measured in the scintillation counter.

RESULTS AND DISCUSSION

Reaction of the N-Terminal Residues. Glucose slowly bound the reactive amino groups of insulin at 37 °C and 68% relative humidity. The average number of

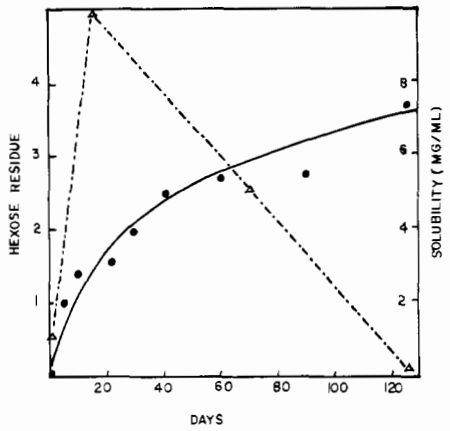

Figure 2. The binding of hexose residues and its effect on the acid solubility of insulin vs. the time of storage with glucose. The initial or normal solubility (approximately 1 mg/ml) rose about ten times but then decreased to less than 0.1 mg/ml after 4 months of storage: solubility (△–·–△); hexose residues (•–•).

hexose residues bound per molecule was determined from the radioactivity recovered in the insulin band of the gel filtration pattern shown in Figure 1. This pattern and the amount of bound ^{14}C were the same whether the brown sample was dissolved and eluted with 8% bicarbonate buffer (pH 7.5) or a 5% acetic acid solution (pH 2.6), thus demonstrating the stability of the Maillard product at low pH.

As indicated in Figure 2, the first effect of the aminocarbonyl reaction was a sudden increase in the acid solubility of insulin. Since only 1.4 hexose residues bound after 15 days, the attack at one or two strategic sites of the polypeptide appeared sufficient to shift the hexamer–dimer equilibrium toward the formation of acid-soluble dimers, resulting in such a sizable increase of solubility. Analysis of the free amino groups of the protein by dansylation revealed that 16.8, 33.3, and 47% of the initially reactive B_1-phenylalanine, A_1-glycine, and B_{29}-lysine (+ B_{22}-arginine), respectively, remained unreacted after 15 days of reaction. In the insulin crystal, neither of these reactive residues is known to be involved in monomer–monomer contacts. The B_1-phenylalanine residue, however, is known to participate in five dimer–dimer contacts which can be essential in maintaining the hexamer organization (Blundell et al., 1972). Considering that the average number of sugar residues bound to one molecule of insulin at 15 days of reaction was between 1.40 and 2.02, it can be concluded that reaction at B_1-phenylalanine and A_1-glycine was the principal reason for the disruption of the insoluble hexamer. Marcker (1960) showed that hexamer formation was impaired with such large substituents as phenylcarbamyl at B_1.

Although dansylation of the unreacted amino groups revealed that the total number of reacted residues was 2.02 vs. 1.4 by the measurement of ^{14}C binding, this method confirmed the order of reactivity reported by Schwartz and Lea (1952), i.e., $B_1 > A_1 > B_{29}$. The results also emphasized the participation of the different primary amines of insulin in the reaction, indicating that no polymerization of the sugars was taking place at any one amino group as in the case of Clark and Tannenbaum's (1974) insulin. The extent of the reaction of B_{22}-arginine with glucose was considered unimportant at 15 days because of the high pK of the guanidinium group, and the participation of this residue in a salt bridge with the A_{21}-carboxylate forming an essential bond in the functional structure of the monomer (Blundell et al., 1972).

Table I. Biological Assay of Maillard Insulin after Binding an Average of 1.4 Sugar Residues per Monomer Molecule[a]

Blood glucose depression (rabbit assay), %		Blood Trp elevation (rat assay),[b] μg/ml		
Normal potency	Maillard insulin potency	No treatment	Normal insulin	Maillard insulin
100	78.8	22.46 ± 3.4	30.18 ± 7.3	31.33 ± 3.7

[a] Six rabbits were used for blood glucose and 24 young rats for blood tryptophan. [b] Comparison of means from rat assay: normal × Maillard: $n = 7; t = 0.370$; no significance; no treatment × Maillard: $n = 7; t = 4.089$; $P < 0.01$; no treatment × normal: $n = 7; t = 2.523$; $P < 0.05$.

Biological Functionality of Maillard Insulin. While disruption of the quaternary structure of the hormone produced a marked change in one of the physicochemical properties of the crystal, namely the increase in solubility, the tertiary structure of the monomer suffered little or no alterations according to the biological assays. As shown in Table I, the potency of insulin in rabbits decreased by 21.2% after 15 days of reaction but no change was observed in its ability to raise the level of serum tryptophan in young rats. The loss of some biological activity was expected to result from some distortion of the tertiary structure rather than the blocking of the α- and ε-amino groups. Fraenkel-Conrat and Fraenkel-Conrat (1950) and Lindsay and Shall (1971) already demonstrated that unsubstituted α- and ε-amino groups were not a requirement for the blood glucose-lowering activity. Introduction of bulky substituents into these groups, nevertheless, can result in reduced activity due to conformational changes (Levy and Carpenter, 1967). Structural alterations after 15 days of reaction with glucose obviously were insufficient to prevent normal interaction of the hormone with serum albumin in the rat.

Biological Stability of Maillard Insulin. It is difficult to assess the in vivo stability of chemically modified insulins in general because of the minute hormone concentration tolerated by the animal. Maillard insulin of high specific activity ([^{14}C]glucose) incubated for 2 h with a rat kidney homogenate containing active deacylases yielded no increase in the level of Cl_3CCOOH soluble radioactive material. Although some soluble radioactivity did appear after 4 h of exposure to the homogenate, this suggested that the Maillard product could be stable in the bloodstream for the duration of the assays.

Inactivation of food proteins by the Maillard reaction could be explained by the inability of the protein to release essential amino acid residues due to either formation of resistant cross-linkings or substitution of enzyme-binding groups (steric hindrance). A clear demonstration of this mechanism is provided by the isolation of a limit peptide from insulin browned at 55 °C and subjected to the action of mixed proteases (Clark and Tannenbaum, 1974).

According to our results with insulin, however, such a mechanism of inactivation could not be so evident at the early stages of the Maillard reaction. When the reaction was allowed to occur at 37 °C, at least three features were observed: (a) the N-terminal residues reacted more readily than other amino groups in the protein, (b) nitrogen monosubstitution by the hexose was most likely a first step toward polymerization and cross-linking, and (c) possible rapid and significant alteration of a protein's quaternary but little alteration of its tertiary structure could be expected before the onset of polymerization. There is no doubt that polymerization and cross-linking can eventually take place at the lower temperatures as well. In fact, insulin samples from our experiment stored with [^{14}]-glucose for 1 year yielded an insoluble, deeply brown mass.

A new mechanism may be needed to explain the initial loss of biological value of egg albumin, and perhaps other food proteins, which have reacted with glucose under conditions too mild to expect any cross-linking. Although no direct comparison could be made between insulin and the proteins present in egg albumin, it is realistic to predict little biological inactivation of a food protein that has reacted with one or two sugar residues per polypeptide chain. In order to explain the high loss of biological value in the egg albumin of Tanaka et al. (in press), perhaps some thought should be given to the existence of weakly bound nonprotein components which can be irreversibly lost early in the reaction.

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