Biological Inactivation of Proteins by the Maillard Reaction

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BIOLOGICAL INACTIVATION OF PROTEINS BY
THE MAILLARD REACTION

By
JAIME AMAYA-FARFAN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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DOCTOR OF PHILOSOPHY DISSERTATION

OF

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ABSTRACT

The effects of the Maillard reaction on the biological activity of insulin as a hormone, and on the utilization of egg albumin as a source of amino acids have been studied.

In order to know the number, the position, and the effect of the glucose residues that bind a protein, crystalline zinc insulin was stored with $^{14}$C-glucose at $37^\circ$ and 68% R.H. for four months. The unreacted sugar was separated from insulin in a Bio-Gel column and the bound radioactivity counted. The first sugar residue reacted within five days, and additional 2.7 residues reacted through the end of the storage. Binding of 1.4 residues increased the acid solubility of insulin ten times suggesting impairment of hexamer formation. The ability of this Maillard insulin in controlling the blood glucose and tryptophan levels suffered little or no change. After four months the solubility decreased by a factor of 1000.

Since the $\alpha$-amino groups are more reactive than any other group in a protein, the N-terminal Maillard reaction was studied using two dipeptides. The reaction of glycyl-L-leucine with an excess of glucose gave a compound which, after chromatographic purification, was tentatively identified as N, N-di(1-deoxy-2-ketosyl)-glycyl-L-leucine. This Maillard product was not hydrolyzed by leucine-amino peptidase. In vivo, the compound was completely unabsorbed from
the intestine of duodenum/portal vein cannulated rats, indicating that the residue adjacent to the N-terminal amino acid of this Maillard dipeptide was not released and transported even if it did not directly participate in the Maillard reaction. From the portal blood levels of free tryptophan, it was learned that the various N-substituted dipeptides present in an L-lysyl-L-tryptophan premelanoidin mixture may be hydrolyzable and serve as a source of tryptophan without interfering with the absorption of the normal dipeptide.

The exclusion of small, soluble peptides from absorption suggested that they may be detected in the feces. The soluble fraction of the feces of rats fed Maillard egg albumin was analyzed, after extraction and filtration with solutions of (i) sodium chloride, (ii) ammonium acetate, and (iii) pyridine acetate. The NaCl extracts showed the accumulation of ninhydrin-positive bands of about (I) 1300, (II) 1000, and (III) 800 molecular weight, all exhibiting browning characteristics. The first two bands contained high proportions of lysine, plus other essential and non-essential amino acids. Band III yielded 90% NH₃ upon hydrolysis. (ii) Portions from the ammonium acetate extracts, containing bands I and II, were resolved into six peaks by cation exchange and analyzed for amino acids. (iii) Samples treated with pyridine acetate gave a profile different from that in (I). Cation exchange chromatography revealed some fifteen peaks, which were neither peptides nor glycosidic residues. Maillard
ovalbumin tagged with either $^{14}$C-lysine or -tryptophan was fed to rats to assess the fecal recovery. Feeding the Maillard ovalbumin raised the recovery of radioactivity from 1 to 7% for the former, and from .2 to 3% for the latter. Such loss was not considered significant, and thus other possibilities are suggested to account for the drastic reduction of nutritive value of mildly brown egg albumin.
DEDICATION

To my parents, Julio C. and A. Rita, who urged me to stay in school. This thesis is also dedicated to my wife, Delia, for her moral support.
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The body of this thesis comprises three manuscripts, which are written to meet the guidelines of two different journals, and in a manner that is acceptable also by the Graduate School. The first manuscript deals with the reaction between crystalline insulin and glucose, and the effect of the resulting modification on two of the hormone's biological functions. The second manuscript is a study of the effect of sugar-substitution of the \( \alpha \)-amino group of a dipeptide on the peptide bond hydrolysis and intestinal absorption of the products. The third paper focuses on the accumulation of some low-molecular weight nitrogenous compounds in the feces of rats as a result of feeding egg albumin which had been stored with glucose.

The underlying element of the three manuscripts is the effect of the heat-catalyzed reaction of glucose with the amino groups of proteins (Maillard reaction) on the structural and physiological properties of a protein; be it of a pharmacological or nutritional importance. More specifically, the author is interested in establishing the number of sugar residues that can react with the \( \alpha-, \varepsilon- \) or any other kind of amino groups of a protein. Some authors have proposed, as late as 1968, that all the peptide nitrogens can bind glucose, but there are basic theoretical considerations that argue against this possibility. To try to answer this I chose a well-defined system such as insulin.
Second, we need to know the importance of the sugar-substituted \( \alpha \)-amino terminus in the hydrolysis and absorption of, not only the terminal amino acid, but also those residues adjacent to it. Something is known on the fate of a few amino acid-sugar compounds when they reach the intestine, but extension of these results to proteins would be premature. Therefore, I chose to study the absorption and hydrolysis of glycyl-L-leucine and L-lysyl-L-tryptophan after allowing them to react with glucose.

Lastly, a proof is needed for the accumulation of small natural peptides in the feces of rats fed Maillard egg albumin. If the hypothesis is correct that Maillard proteins are hydrolyzed down to fragments of a certain size and that some of these fragments are not absorbed because of the lack of free \(-\)amino groups, then such fragments may be found excreted in the feces.

There is a second part to this thesis which is a series of three appendices designed to provide supplementary information to the reader. Appendix A contains the chemical principles of the Maillard reaction. It also points out some of the early conceptions and misconceptions that came with the development of this field of research. Appendix B contains a detailed description of a surgical technique for the \textit{in vivo} study of intestinal absorption in rats. The major advantage of this technique is that it allows the researcher to use every animal as its own control and employ small amounts of substrate. The procedure is the result of
both the first-hand knowledge acquired from its initiator, Dr. Hugo Gallo-Torres of Hoffman-LaRoche Laboratories, and a modification that is a result of my own experience. Appendix B also describes some buffer gradients which were useful in the chromatographic separation of the fecal peptides. The conditions for a modification of the p-dimethylaminobenzaldehyde in the determination of tryptophan and phenol are also recorded in this appendix. Appendix C is the summary and significance of the conclusions as they would appear in the classical thesis format.
EFFECT OF THE MAILLARD REACTION ON THE BIOLOGICAL ACTIVITY OF INSULIN

ABSTRACT

Crystalline zinc insulin was stored with 1% glucose (1:53 mole ratio) at 37° and 60% R.H. to undergo the Maillard reaction for four months. The unreacted sugar was separated from the insulin and found to have increased radiactivity. Insulin-carbohydrate residue reacted with the polypeptide within the first five days of storage. Throughout the rest of the incubation period additional 2.7 residues bound the hormone. The binding of an average of 1.4 hexose residues, at fifteen days of storage, brought about a ten-fold increase in the acid solubility of insulin, thus suggesting that the modification impairs hexamer formation. By a simultaneous, yet slower, process the solubility was observed to increase by a factor of about 1000 after four months of storage. The sixteen-day Maillard insulin had lost 22% of its potency while its ability to raise the level of blood tryptophan in young rats has not changed.
ABSTRACT

Crystalline zinc insulin was stored with $^{14}$C-glucose (1:55 mole ratio) at 37° and 68% R.H. to undergo the Maillard reaction for four months. The unreacted sugar was separated from insulin in a Bio-Gel column and the bound radioactivity counted. On the average, one carbohydrate residue reacted with the polypeptide within the first five days of storage. Throughout the rest of the incubation period additional 2.7 residues bound the hormone. The binding of an average of 1.4 hexose residues, at fifteen days of storage, brought about a ten-fold increase in the acid solubility of insulin, thus suggesting that the modification impairs hexamer formation. By a simultaneous, yet slower, process the solubility was observed to decrease by a factor of about 1000 after four months of storage. The fifteen-day Maillard insulin had lost 22% of its potency while its ability to raise the level of blood tryptophan in young rats had not changed.
INTRODUCTION

In the Maillard reaction, reducing sugars form condensation products with the amino groups of amino acids, peptides and proteins. At the initial stages, $\alpha$-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 with proteins has been mostly studied on proteins used for food, which appear to lose considerable solubility, digestibility and biological value along the course of the reaction.

As a result of working with undefined protein systems, it has not been possible to ascertain (a) which amino acid residues besides the terminal amino and lysine in the polypeptide chain directly participate in the reaction; (b) to what extent do the physiochemical changes impede the functionality of a Maillard protein, and (c) how many hexose residues react per molecule of protein. The only attempt to use insulin as a model protein for the Maillard reaction was made by Schwartz and Lea (1951), who estimated the radioactivity of the $\alpha$- and $\epsilon$-amino groups with glucose but did not evaluate the product in terms of biological function.

The objective of this work was to modify crystalline insulin by the Maillard reaction, measure the average number of hexose residues bound to the protein, and estimate the effect of any physiocochemical changes on two different biochemical functions.
MATERIALS AND METHODS

The Browning of Insulin. Reaction mixtures were prepared in 20-ml glass vials suspending 50 mg of crystalline insulin (bovine pancreas, .5% zinc, Sigma Chemical Co.) in 10 ml of 7.75% glucose. The mixtures were lyophilized using minimum heat and time. The vials were then stored uncapped in sealed chambers at 37° and 68% R.H. for up to four months. For the glucose binding studies, each vial also contained from 25 to 50 μCi of UL 14C-D-glucose (International Chemical & Nuclear Co., Irvine, California).

Glucose Binding. For every time point, the contents of a vial were dissolved in 10 ml of an acetic acid solution (pH 2.6), and .3 ml loaded onto a Bio-Gel P-6 (200-400 mesh, Bio-Rad Laboratories, Richmond, California) column, and eluted with the same acetic acid solution. Other conditions for the gel filtration were: Column size, 1 x 50 cm; flow rate, 22 ml/hr; void volume, 18 ml; fraction volume, 1.5 ml.

Fifty microliters of each fraction were counted in a liquid scintillation counter (Nuclear Chicago, Mark I) using a fluorescent solution made by mixing 2 l of toluene, 2 l of 1,4-dioxane, 1.2 l of ethanol, 260 g of napthalene, 26 g of PPO, and .5 g of POPOP. The ninhydrin pattern was obtained after hydrolysis of .15 ml of each fraction with 1 ml of 2.5 N KOH at 121° for 40 minutes, followed by neutralization with .6 ml of 30% acetic acid. Unreacted glucose was determined in
the fractions by the Glucostat method (Worthington Biochemicals, Freehold, New Jersey).

The glucose-free Maillard insulin was dansylated and analyzed according to the procedure of Gros and Labouesse (1969), except that (a) 10 microliters containing 10-15 nmole of either Maillard or normal insulin were used; (b) the urea was Sequanal grade from Pierce Chemicals (Rockford, Illinois); (c) the TLC plates were spotted, developed and scraped promptly to avoid loss of fluorescence. The material collected from the spots was read 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 fifteen days. The unreacted sugar was eliminated by washing with distilled water and centrifuging three times. This procedure afforded 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 (Glochester Rabbitry, Glochester, Rhode Island) assay (US Pharmacopeia), and it was further tested in its ability to raise the blood tryptophan levels in young rats (Charles River Breeding Laboratories, Wilmington, Massachusetts) as described by 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 m Ci/m mole) was
incubated with a rat kidney homogenate (Price and Greenstein, 1948) at 37° for 0, 1, 2 and 4 hours. The reaction was stopped with 1.5 volume of 10% TCA, the precipitate centrifuged and the supernatant counted for radioactivity.

The reaction was incubated with a rat kidney homogenate (Price and Greenstein, 1948) at 37° for 0, 1, 2 and 4 hours. The reaction was stopped with 1.5 volume of 10% TCA, the precipitate centrifuged and the supernatant counted for radioactivity.

The binding of glucose to insulin as a function of storage time is presented in Figure 1. As five days after storage, an average of 0.96 glucose residues had bound the hormone with a resulting increase in solubility of about ten times. After fifteen days of storage, the solubility of Maillard insulin was observed to decrease. At four months, an average of 3.7 glucose residues had bound. More acid (pH 2.4) was needed to bring the Maillard insulin to its solubility.

Insulin that had been stored with glucose for fifteen days was separated from the unreacted glucose and then assayed for available amino groups and biological activity. Analysis of the hydrolysate products of the deamidation reaction indicated that 15.8, 35.5 and 67 per cent reacted available of the initially reactive amino groups in
RESULTS

In the dry state (16% water), glucose slowly binds the reactive amino groups of insulin by the Maillard reaction at 37° and 68% R.H. The average number of 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 radioactivity were the same whether the brown sample was dissolved and eluted with bicarbonate buffer (pH 7.5) or acetic acid (pH 2.6) thus demonstrating the stability of Maillard insulin at low pH.

The binding of glucose to insulin as a function of storage time is presented in Figure 2. At five days after storage, an average of .98 glucose residues had bound the hormone with a resulting increase in solubility of about ten times. After fifteen days of storage, the acid solubility of Maillard insulin was observed to decrease. At four months, when an average of 3.7 glucose residues had reacted, more acid (pH 2.4) was needed to bring the Maillard insulin into solution.

Insulin that had been stored with glucose for fifteen days was separated from the unreacted glucose and then assayed for available amino groups and biological activity. Analysis of the hydrolysis products of the dansylation reaction indicated that 16.8, 33.3 and 47 per cent remained available of the initially reactive amino groups in
phenylalanine B1, glycine A1, and lysine B 29 + arginine B22, respectively. The extent of the reaction of arginine B22 with glucose could not be determined with the TLC system used for the experiment. The dansylation reaction appeared to give higher values of glucose-reacted amino groups but this was most likely due to lower recoveries of fluorescence from the TLC spots containing small amounts of dansyl phenylalanine and dansyl glycine. The biological assays (Table 1) showed that the blood-glucose depressing activity of the Maillard insulin was reduced by about 20 per cent while the blood-tryptophan elevating activity remained unchanged.

When Maillard insulin of high specific $^{14}$C-activity was incubated with a rat kidney homogenate at 37°C, there was no release of TCA-soluble counts before two hours. At four hours of incubation, however, some radioactive supernatant was already evident but this could have been greatly promoted by proteolytic action.
The diagram shows the concentration of substances in a given fraction of a tube. The peaks indicate the presence of substances at specific fractions of the tube. The x-axis represents the fraction number, and the y-axis represents the concentration. This graph is used to analyze the distribution of substances in a chromatographic separation process.
Figure 1. Bio-Gel P-6 column separation of unreacted and insulin-bound hexose. A typical load consisted of .257 mole of insulin and 6.177 u mole of originally $^{14}$C-D-glucose (s.a. $3.2 \times 10^6$ dpm/mg) in .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. The area under the radioactive peaks was integrated and the number of bound residues calculated from the radioactivity under the insulin peak. Recoveries were usually better than $100 \pm 6\%$. Ninhydrin (●-●); Glucostat (○-○); radioactivity (x-x).
Figure 2. The binding of hexose residues and its effect on the acid solubility of insulin versus the time of storage with glucose. The initial solubility (app. 1mg/ml) rose about ten times but then decreased to the level of ug/ml after four months of storage.
FIGURE 2
**TABLE 1**

Biological assay of Maillard insulin after binding an average of 1.4 sugar residues per monomer molecule

| Blood glucose depression (Rabbit assay) | Blood tryptophan elevation (Rat assay) |
|----------------------------------------|---------------------------------------|
| **Normal potency** | **Maillard ins. potency** | **No treatment (micro G/ML)** | **Normal (micro G/ML)** | **Maillard insulin (micro G/ML)** |
| 100% | 78.8% | 22.46 ± 3.4 | 30.18 ± 7.3 | 31.33 ± 3.7 |

Normal * Maillard: N = 7; T = .370; N.S.
--- * Maillard: N = 7; T = 4.089; P .01
--- * Normal: N = 7; T = 2.523; P .05
DISCUSSION

The temperature-dependent reaction of D-glucose with the primary amino groups of insulin, which yields the moderately stable N-1-deoxy-D-fructosyl derivatives, may prevent the formation of the hexamer in solution. The relative ease with which glucose attacks the amino groups of insulin B1 Phe was suggested by Schwartz and Lea (1951), who reported that, at four days of reaction, 75 per cent of the B1 Phe residue had reacted compared to 37 per cent of the A1 Gly or 20 per cent of the B29 Lys residues. By the dansylation reaction we found that the total number of amino groups reacted after fifteen days with glucose was 2.02. Although this was in disagreement with the value of 1.4 obtained from the binding of 14C-glucose, dansylation of Maillard insulin corroborated the observation by Schwartz and Lea that the order of reactivity is B1 > A1 > B29. In the crystal, neither of these reactive residues is known to be involved in monomer-monomer contacts. The Phe B1 residue is known, however, to participate in five dimer-dimer contacts which can be essential in maintaining the hexamer organization (Blundell and Assoc., 1972). Considering that the fast increase in acid solubility noticed after five days of incubation coincided with the binding of about one glucose residue, it is conceivable that formation of N-1-deoxyfructosyl Phe B1 is principally responsible for the failure of dimers to associate into soluble
polymers at pH 2.6. Marcker (1960) showed that hexamer
formation was impaired with large substituents such as
phenylcarbamyl at Bl. The extent of the reaction of B22 Arg
with glucose was considered unimportant at fifteen days be-
cause of the unreactivity of the guanidinium group at neutral
pH, and the participation of B22 in a salt bridge with the
A 21 carboxylate forming an essential bond in the functional
structure of the monomer.

While the solubility of the hormone at pH 2.6 was
markedly increased by mild browning, the biological activity
was decreased only slightly. By examining the biological ac-
tivity of acetylated insulin, it was proposed that unsubstitu-
ted α- and ε-amino groups were not required for the blood-
glucose lowering effect of insulin (Fraenkel-Conrat and
Fraenkel-Conrat, 1950; Lindsay and Shall, 1971). The intro-
duction of bulkier substituents at the amino groups resulted
in reduced activity perhaps due to considerable distortion of
the tertiary structure rather than to the blocking of these
groups (Levy and Carpenter, 1967). Despite its relatively
large size, glucose can approach and readily react with the
Bl terminal amino group. The α-N-1-deoxy-2-ketohexose sub-
stituent, though bulky enough to cause the dislocation of
dimer-dimer contacts, did not appear to produce any major
distortions in the tertiary structure of the monomer as in-
ferred from the results of the potency assay. Furthermore,
the fifteen-day Maillard insulin exhibited the same activity
of normal insulin in raising the blood-tryptophan level in
rats suggesting again that the Bl and, perhaps, the Al and B29 primary amino groups are not directly involved in this function.

The N-deoxyfructosylinsulin was stable at $4^\circ$ in acidic pH for at least a week but its stability was not tested in vivo. It is known, however, that radioactive tryptophan is not available for protein synthesis if it is injected intravenously in the form of N-deoxyfructosyltryptophan (Sgarbieri and Assoc., 1973). Since the in vitro test showed that Maillard insulin is apparently stable for two hours in the presence of kidney deacylases, it was assumed that the N-glycosidic bond was resistant to enzymatic hydrolysis in vivo for the duration of the biological assays.

When insulin is mixed with glucose and stored in the dry state at mild temperatures, approximately four sugar residues will bind the hormone in a period of four months suggesting that monosubstitution of the reactive amino groups may take place at the initial stages of the reaction. The acid solubility, which increased about ten times with the binding of an average of 1.4 residues, was observed to progressively decrease upon further reaction with hexose. Impairment of hexamer association in solution was the probable reason for the initial increase of solubility, while significant monomer distortion could have accounted for the subsequent loss of solubility. The insulin-mediated sugar metabolism in rabbits was somewhat decreased but no change was detected in the ability to raise blood tryptophan in rats as a result of
storing the hormone for fifteen days. Such observations contrast with the effects of the reaction on food proteins, which have been reported to lose up to 50 per cent of their biological value only after ten days of storage under identical conditions (Tanaka and Assoc.).
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THE INTESTINAL ABSORPTION OF N-1-DEOXY-2-KETOSYL SUBSTITUTED DIPEPTIDES
ABSTRACT

The importance of free $\alpha$-amino groups in the hydrolysis and absorption of peptide-bound amino acids has been studied in connection with the Maillard reaction of two dipeptides. The reaction of glycyl-L-leucine with an excess of glucose in methanolic medium gave mostly a compound which, after chromatographic purification, was tentatively identified as N, N-di(1-deoxy-2-ketosyl)-glycyl-L-leucine. The Maillard product was shown not to be a substrate for leucine-amino peptidase. In vivo, the compound was completely unabsorbed from the intestine of duodenum/portal vein cannulated rats, indicating that the residue adjacent to the N-terminal amino acid of this Maillard dipeptide was not released and transported even if it did not directly participate in the Maillard reaction. Moreover, the presence of Maillard dipeptide did not appear to inhibit the absorption of normal glycyl-L-leucine. At least five different products were detected from the reaction of L-lysyl-L-tryptophan with glucose (1:1 amino-to-carbonyl ratio). From the portal blood levels of free tryptophan it was learned that the various N-substituted dipeptides in the premelanoidin mixture may be hydrolyzable and serve as a source of tryptophan. The presence of premelanoidins in the intestine did not appear to preclude hydrolysis and absorption of the normal dipeptide. Traces of pyridine in some preparations were suspected of inhibiting hydrolysis of the dipeptide.
INTRODUCTION

Some $\alpha$-N-acetylated amino acids are absorbable and utilizable substrates in bacteria but not necessarily in the small intestine of mammals. Bacteria can absorb and utilize $\alpha$-N-acetyllysine (Payne and Gilvarg, 1971). N-acetyl-L-tryptophan that was fed to or injected into rats was demonstrated to produce normal growth after deacetylation in the body (du Vigneaud et al., 1932). Neuberger and Sanger (1943) showed that $\alpha$-N-acetyllysine fed to rats had no nutritional value whereas the $\varepsilon$-N-acetyl derivative did to some extent.

Substitution of the $\alpha$-amino function of amino acids with larger groups such as 1-deoxy-2-ketohexose, as it occurs in the Maillard reaction of amino acids with glucose, makes the amino acid generally unavailable to rats (Sgarbieri et al., 1973a). About 85% of the N-1-deoxyfructosyl derivative of leucine was shown to remain unabsorbed in the intestine of rats three hours after stomach-intubating the compound. A similar fate was suggested for N-1-deoxy-fructosyl-L-tryptophan (Sgarbieri et al., 1973b).

While in bacteria the structural requirements for oligopeptide absorption have been worked out to some detail, virtually nothing is known about the role of the peptide functional groups or structure in intestinal absorption. In bacteria, the terminal amino group of an oligopeptide must be unacylated for absorption. For instance, E. coli cannot
absorb either $\alpha$-N-acetyldilysine or $\alpha$-N-acetyltetralysine, and $\alpha$-N-acetyltrilysine is only partially absorbed (Payne and Gilvarg, 1971). Although lysine is the only residue within a polypeptide chain which has been proven to react with glucose (or a reducing sugar) during the mild Maillard reaction of proteins, resulting in a critical loss of nutritive quality, the terminal $\alpha$-amino groups appear to react more readily. Therefore, it is of interest to learn if the N-terminal oligopeptides produced in the animal digestion of Maillard proteins can be degraded and utilized down to the terminal, N-substituted residue.
EXPERIMENTAL

Preparation of \(\alpha\)-N-fructosyl glycylleucine

All materials were reagent grade. The procedure used was an adaptation of that employed by Abrams et al. (1955) for the synthesis of fructose-L-leucine. Glycyl-L-leucine (.9137 g; Schwarz-Mann, Orangeburg, New York) and glucose (13.2 g; Fisher Scientific) were dissolved in 540 ml of methanol and refluxed for five hours. The mixture was concentrated in a flask evaporator, loaded onto a Dowex 50W x-4 column (6.5 x 43 cm), washing with 4 l of water and then with successive 4 l-steps of trichloriacetic acid (TCA) .05 M, .10 M, .10 M, .2 M, .2 M solutions. Other conditions for the column were: Flow rate, 700 ml/hr; void volume, 542 ml; fraction size, 250 ml.

Since the amino-sugar bond is labile at 100\(^\circ\), the fractions were tested for both a ketose compound by the ferricyanide reaction (Borsook et al., 1955), and an amino acid by the ninhydrin reaction (Moore and Stein, 1948). Three bands were eluted between fractions 45 and 80 including a major ferricyanide/ninhydrin-positive band about fraction 76. Although weakly positive to the ninhydrin test, the first band was negative in the amino acid analyzer. The second band was also discarded on the basis of its low ninhydrin color.

The fractions of the large peaks which accounted for about 80-90% of the total ninhydrin positive material, were
pooled into two aliquots and each extracted seven times with ether; the ether was removed in a flask evaporator, and the product lyophilized. Each aliquot was dissolved in 50 ml of water, followed by repetition of the ether extraction evaporation and lyophilization steps. In the amino acid analyzer (Technicon Auto Analyzer) the compound was eluted in a single band near methionine while the unreacted dipeptide chromatographed between tryosine and ammonia. Upon acid hydrolysis, the product showed equimolar amounts of glycine and leucine whose proportion to total input suggested the compound was disubstituted. There was no detectable TCA residue as monitored in a GLC equipped with a free fatty acid column.

Preparation of Maillard lysyltryptophan

The dipeptide was browned in two different ways. Two aliquots of 135 μ mole of L-lysyl-L-tryptophan acetate (Schwarz-Mann, Orangeburg, New York) were dissolved with D-glucose (Fisher Scientific) at 2:1 molar ratio in a small volume of water and lyophilized. One of the aliquots was stored at 37°, 68% R.H. for 30 days and the other kept at -20°. Some of the products of the premelanoidin mixture could be separated either by active carbon (Fisher Scientific) or a cellulose powder (Whatman CF11) column eluted with water-saturated butanol. Alternatively, 500 mg of dipeptide were refluxed with 4 g of glucose in 65 ml of methanol for four hours. The volume was reduced to 4 ml in a flask evaporator, the pH adjusted with HCl to 3, and 2 ml loaded onto
a Dowex 50W x-4 (110 x 2.5 cm) column. The elution began with .25 l of .2 M pyridine acetate (pH 3.25) followed by 1.5 l of .4 M (pH 5) and 1.5 l of 2 M (pH 5). Other conditions were: Flow rate, 120 ml/hr; void volume, 180 ml; fraction size, 10 ml. The fractions were tested as described in the preceding section by the ninhydrin and the ferri-cyanide reactions. Although three peaks were found about fractions 36, 60 and 93, only the material in the last peak was collected since it contained most of the double-positive compound. The bulk of the buffer was removed by repeated flask evaporation and the concentrated material was repeatedly lyophilized to remove traces of pyridine. The product, however, was not assumed to be pyridine free.

In vitro hydrolysis of fructosyl glycylleucine

Leucine amino peptidase (LPA, E.C. 3.4.1.1, hog kidney Worthington Biochemicals, Freehold, New Jersey) was activated for one hour as described in the manufacturer's manual (Worthington Enzyme Manual, 1972; also Mitz and Schlueter, 1958). The normal and brown peptides were conveniently dissolved at 1/3 of the recommended concentration, the molecular weight of fructosyl glycylleucine being taken as 350.3.

For the hydrolysis reaction, the dipeptide and an equivalent concentration of glycine + leucine were correspondingly placed in the sample and reference cuvets of a double-beam spectrophotometer (Beckman DB-G). The difference in absorbance at 238 nm was usually between .45 and .65.
Fifty microliters of freshly activated LPA were added to the substrate and the decrease in absorbance at 238 nm was recorded every five minutes. Since Maillard compounds absorb rather strongly in the UV range, it was necessary to adjust the absorbance of the Maillard substrate prior to addition of the enzyme by adding drops of brown glucose or fructose (browned on a hot plate for five minutes) to the reference cuvet. When the Maillard substrate was exposed to the UV light, the absorbance was occasionally observed to rise slightly (first five minutes). In such cases, the substrate was allowed to stand in the spectrophotometer without the enzyme until the rising trend ceased.

**In vivo absorption of Maillard peptides**

Female Sprague-Dawley rats of 170-200 g (Charles River Breeding Laboratories, Wilmington, Massachusetts), previously fasted for fifteen hours, were catheterized in the duodenum and the hepatic portal vein by the fast, direct cannulation method described by Gallo-Torres and Ludorf (1974) (see Appendix B). One hour after recovery from the anesthesia, the rats were injected .25 m mole of either normal or Maillard dipeptide in 1 ml saline of pH about 8.

In the case of Maillard glycylleucine .25 ml blood samples were withdrawn into heparinized, siliconized centrifuge tubes at different times after injection of the substrate. The separated serum was deproteinized with 2 volumes of 10% TCA followed by centrifugation at 15,000 x g.
The TCA was ether-extracted in the same tubes, the aqueous phase dried in a flash evaporator, and the redissolved product loaded onto an amino acid analyzer (Technicon Auto Analyzer) for the quantitative determination of both glycine and leucine.

For the absorption of Maillard lysyltryptophan, 80 microliters of blood were withdrawn into heparinized capillaries, spun in a hematocrit, and the free tryptophan in the serum determined by method of Denckla and Dewey (1967) (see Appendix B). A .20 ml serum sample was also deproteinized as indicated above and chromatographed in the amino acid analyzer using a special gradient (see Appendix B) to determine the influx of unhydrolyzed normal and brown peptides.
RESULTS

The major Maillard product of reacting glycyl-L-leucine with an excess of glucose chromatographed as a single peak near methionine in the amino acid analyzer. Upon acid hydrolysis, equimolar amounts of glycine and leucine were obtained whose ratio to the total weight suggested the compound was $\alpha$-N,N-di-(1-deoxy-2-ketohexosyl)-glycyl-L-leucine (Abrams et al., 1955; Heyns and Noack, 1964).

The premelanoidins formed during the reaction of L-lysine-L-tryptophan with glucose in the dry state were found to contain five ninhydrin-positive peaks plus unreacted dipeptide when chromatographed in an amino acid analyzer using a special gradient (Figure 3). Peaks A through D could be selectively adsorbed onto activated carbon leaving equal amounts of product E and unreacted dipeptide. Passing the mixture through a cellulose column yielded a mixture containing products C and E plus the unreacted dipeptide.

The products from the methanolic reaction present in the major band of the Dowex-pyridine acetate columns were peak C (73%) and unreacted dipeptide (27%), which were also present among the products of the reaction in the dry state.

The Maillard glycyleucine was not hydrolyzable by leucine amino peptidase (LAP in vitro. Figure 4 shows that while no hydrolysis of the brown dipeptide was observed, the enzyme action on normal dipeptide was apparently inhibited
Figure 3. Chromatographic separation of lysyltryptophan from the five major products formed during the reaction with limited amounts of glucose. The reaction was carried out by incubating 135 umole of L-lysyl-L-tryptophan with D-glucose (2:1 molar ratio) at 37°, 68% R.H. for 30 days. The separation was done in a Technicon Auto Analyzer as described in Appendix B.
FIGURE 3
Figure 4. Leucine-aminopeptidase (LAP) hydrolysis of glycylleucine and the reduction of the reaction rate by adding increasing amounts of Maillard glycylleucine. The brown dipeptide was not hydrolyzable by LAP. Addition of Maillard dipeptide to normal glycylleucine almost (1:1 molar ratio) brought the reaction to a halt. The rates were measured at room temperature by taking the difference in UV absorbance between the dipeptide and the hydrolyzed products. The concentration of either substrate, blank or inhibitor was .0416 M. The reaction mixtures were made as indicated in the Worthington Enzyme Manual (1972)
FIGURE 4
in the presence of the brown substrate. Addition of about 10% brown dipeptide to normal dipeptide lowered the rate of hydrolysis by more than 35%. The hydrolysis was arrested as the inhibitor-to-substrate ratio approached 1:2, respectively.

Maillard glycylleucine was not absorbed in vivo but no effect on the absorption of normal dipeptide was apparent. The absorption patterns of the hydrolyzed amino acids is shown in both Figure 5 and Table 2. The sharp increase in both glycine and leucine in the portal blood minutes after duodenal injection of the normal dipeptide was contrasted by the almost total absence of either the free amino acids or the dipeptide after injection of the Maillard peptide. When the brown dipeptide was injected together with regular glycylleucine in a 1:2 molar ratio, respectively, the concentration pattern of free glycine and leucine in the portal blood resembled that of normal absorption. This indicated that although the brown dipeptide was not a substrate in the small intestine, its presence did not impair the hydrolysis and absorption of the regular dipeptide. The absence of either dipeptide from all the chromatograms also suggested that, at the concentration used, the intact dipeptides were not absorbed.

The in vivo absorption of L-lysyl-L-tryptophan is compared in Figure 6 and Table 3 with the absorption of a mixture containing peak E (73%) and unreacted peptide (27%) which had been prepared in methanol and partially purified in pyridine acetate buffer. Twenty-five minutes after
Figure 5. Pattern of the intestinal absorption of glycylleucine (−x−), glycylleucine in the presence of Maillard dipeptide (−v−), and Maillard dipeptide alone (−o−). The portal blood from cannulated rats was deproteinized in 10% TCA and the ether-extracted supernatant was analyzed (Technicon Auto Analyzer) for glycine and leucine at every time-point. For comparison of the glycine and leucine concentrations, see Table 2.
FIGURE 5

GLYCINE (UML/ML PLASMA)

MINUTES

20 40 80 100
**TABLE 2**

Intestinal absorption of glycyl-L-leucine and its Maillard derivative as determined by amino acid residue concentration (u mole/ml) in portal plasma.

| Time (min) | Glycylleucine (g) | Fructosyl glycylleucine (g) | Glycylleucine + Fructosylglycylleucine (g) |
|-----------|-------------------|-----------------------------|-------------------------------------------|
|           | glycine leucine    | glycine leucine             | glycine leucine                           |
| 0         | .28               | .31 ± .01                   | .30                                        |
| 5         | 1.98              | .10 ± .02                   | 2.08                                       |
| 10        | 3.68              | .41 ± .04                   | 3.52                                       |
| 15        | 2.72              | .11 ± .02                   | 2.98                                       |
| 20        | 3.36              | .36 ± .06                   | 1.40                                       |
| 25        | 1.99              | .10 ± .03                   | 1.53                                       |
| 30        | .63               | .43                         |                                            |
| 35        | .85               |                               |                                            |
| 40        | .39 ± .02         | .14 ± .02                   | 1.07                                       |
| 55        | .29               | .39 ± .02                   | 1.79                                       |
| 60        | .35 ± .02         | .13 ± .03                   | .52                                        |
| 75        | .37               | .35 ± .02                   |                                            |
| 80        | .22               | .32                         | .34                                        |
| 90        |                   | .32                         | .41                                        |
| 100       |                   | .30                         |                                            |
| 120       |                   | .29                         |                                            |

1. Blood was sampled from cannulated portal vein, deproteinized and analyzed in a Technicon Auto Analyzer. No dipeptide in either form was detected in blood.

2. One fasted female rat (160 g) was injected with 1 ml of saline containing .125 u mole of neutralized peptide (pH 8).
TABLE 2 (Continued)

3. Two fasted female rats (170 and 200 g) were injected with the Maillard dipeptide as above. Averages appear with standard deviation.

4. One fasted female rat (200 g) was injected with .125 u mole of dipeptide plus .125 u mole of Maillard dipeptide as above.
Figure 6. Pattern of the intestinal absorption of lysyltryptophan (-A-) and the methanol/pyridine-acetate preparation of a Maillard dipeptide mixture (-o-). The mixture contained 27% unreacted dipeptide. The absorption of normal dipeptide was inhibited by .02 M pyridine (-x-). For concentration figures, see Table 3.
**TABLE 3**  
Intestinal absorption of L-lysyl-L-tryptophan and its Maillard derivatives as determined by the free tryptophan concentration (µg/ml) in the portal plasma.

| Time (min) | Lysyltryptophan$^2$ | Maillard dipeptide$^3$ | Lysyltryptophan + brown mixture$^4$ |
|------------|---------------------|------------------------|----------------------------------|
| 0          | 21.93               | 14.45 ± 2.19           | 16.0                             |
| 5          | 43.35               | 20.80 ± 4.24           |                                  |
| 10         |                     |                        | 688.5                            |
| 15         | 82.87               | 23.84 ± .07            |                                  |
| 25         | 103.27              |                        |                                  |
| 30         |                     |                        |                                  |
| 45         | 94.86               | 14.10 ± 1.70           | 2881.5                           |
| 50         |                     | 14.50 ± 2.26           | 3162.0                           |
| 60         |                     |                        |                                  |
| 65         | 59.92               | 14.65 ± 2.47           |                                  |
| 70         |                     |                        |                                  |
| 80         |                     |                        |                                  |
| 85         | 39.52               |                        | 2713.2                           |
| 90         |                     |                        |                                  |
| 100        |                     |                        | 2284.0                           |
| 105        | 30.60               |                        |                                  |
| 110        |                     | 17.00                  | 2422.5                           |
| 120        |                     |                        |                                  |
| 125        | 21.67               |                        |                                  |
| 130        |                     |                        | 17.00                            |
| 250        |                     |                        |                                  |

1. Blood was sampled from cannulated portal veins and analyzed for free tryptophan by the fluorescent method of Denckla and Dewey (1967). Both the normal and a form of the Maillard dipeptide were chromatographically detected in the plasma.
2. One fasted female rat (175 g) was injected with 1.0 ml of saline containing .125 m mole of L-lysyl-L-tryptophan (acetate) of pH about 8.

3. This material, first reacted in methanol and then chromatographed with pyridine acetate, was found to be a mixture of peak C (73%) and reacted dipeptide (27%). Each of the two fasted female rats (165-200 g) were injected with 1 ml of saline containing about .125 m mole of peptidyl residue at pH 8.

4. One fasted female rat (200 g) was injected with 1.0 ml of saline containing .125 m mole of normal dipeptide plus .120 m mole of dipeptide which had been browned with 70 mg of glucose for 30 days.
infusion of L-lysyl-L-tryptophan, the level of free tryptophan in the plasma had risen about five times. The increase was hardly noticeable, however, when the methanolic preparation was infused.

A different result was obtained when all the pre-melanoidins from the reaction in the dry state were infused (Table 3). The level of free tryptophan increased considerably even when the Maillard and the normal dipeptides were close to a 1:1 ratio. Unlike the neutral dipeptide glycylleucine, the basic lysyltryptophan and its Maillard derivative in peak E were observed to diffuse unhdryolyzed into the blood.

To test if the lack of a free tryptophan in the serosal side after infusion of pyridine-acetate preparation was totally or in part due to inhibition caused by pyridine, .25 m mole of untreated L-lysyl-L-tryptophan acetate in .02 M pyridine were infused duodenally. As Figure 6 indicates, the level of free tryptophan increased at about 1/5 the apparent rate of the untreated dipeptide alone. The intact dipeptide was again detected in the serum in concentrations even larger than in the normal case.
DISCUSSION

The major product of the reaction of glycyl-L-leucine with excess glucose was not a substrate for leucine amino peptidase in vitro, and remained unabsorbed in the small intestine of the rat. It appeared therefore that substituting the α-amino group of glycyl-L-leucine with l-deoxy-2-ketohexose blocked the action of an exopeptidase and prevented the in vivo intestinal transport of not only the terminal glycyl, but also the adjacent leucyl residue.

According to the currently accepted mechanism for the absorption of protein hydrolyzates, a protein is not broken down to amino acids by intralumen digestion before the products are taken up and transported across the intestine. Instead, the oligopeptides produced in the liquid-medium digestion are more efficiently adsorbed to class-specific sites, transported inside the mucosal cells, hydrolyzed intracellularly, and the products transported to the serosal side (Matthews, 1971). Meister (1973) has provided a unified concept for amino acid transport which may also explain the uptake, hydrolysis and transport of oligopeptides from the enzymatic point of view. In Meister's model, the amino acid (or the α-amino group of the peptide if the model is extended to peptides) forms an γ-peptide bond with γ-glutamyl transpeptidase. Subsequently, peptides would
undergo hydrolysis followed by amino acid release inside the cell, and the active γ-glutamyl transpeptidase is regenerated by three energy-dependent enzymatic steps. The final transport out of the cell which could take place by a similar process is not considered in this model.

In the light of the above considerations, it appears N-1-deoxy fructosylglycyl-L-leucine cannot bind a γ-glutamyl-type transpeptidase, and is thus flatly excluded from transport into the cell and from hydrolysis. If the Maillard peptide ever gained access to the mucosal cell, some kind of competition could have been observed with the normal peptide but this was not the case. Moreover, the absence of intact Maillard peptide in the blood suggested that the unhydrolyzed compound remained in the intestinal lumen.

The massive influx of free tryptophan into the portal blood after infusion of the premelanoidins (Table 3), formed during the browning of L-lysyl-L-tryptophan with limited amounts of glucose, was an indication that the various l-deoxy-fructosyl derivatives of lysine may not interfere with the transfer and hydrolysis of adjacent residues, regardless of the nutritional availability of lysine itself. The transfer and hydrolysis of the normal dipeptide was neither considered to be impaired by the presence of the premelanoidins.

The role and relative importance of the functional groups of L-lysyl-L-tryptophan could not be conclusively interpreted given that (1) the several Maillard products have
not yet been identified, (2) unreacted dipeptide was found to be an impurity in the brown preparation, and (3) both the normal and one derivative of the brown dipeptide were found in the portal plasma. The fact that pyridine inhibits the system by which this dipeptide is hydrolyzed and its products transported is consistent with the observed increase of intact dipeptide in the plasma, perhaps as a result of the competitive passive diffusion. Finally, it should be noted that the use of volatile buffers such as pyridine acetate for the preparation of biological substrates must be avoided, unless the product is further "desalted" by ion exchange chromatography.
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ACCUMULATION OF SOLUBLE NITROGENOUS COMPOUNDS IN
THE FECES OF RATS FED MAILLARD EGG ALBUMIN
ABSTRACT

The soluble fraction of the feces of rats fed Maillard (30 days, 37°, 68% R.H.) egg albumin was analyzed for peptide residues. Solutions of (i) sodium chloride, (ii) ammonium acetate, and (iii) pyridine acetate were tried for extraction and gel filtration of the soluble fraction. (i) The NaCl extracts showed the accumulation of ninhydrin-positive bands of about (I) 1300, (II) 1000, and (III) 800 molecular weight, all exhibiting browning characteristics. The first two bands contained high proportions of lysine, plus other essential and non-essential amino acids. Band III yielded 90% NH₃ upon acid hydrolysis.

(ii) Portions from the ammonium acetate extracts, which were presumed to contain bands I and II, could be resolved into six peaks by cation exchange. Each peak was analyzed for amino acids.

(iii) Samples extracted and gel-filtered in pyridine acetate gave a ninhydrin-positive profile similar to that in (i) but in different proportions. Cation exchange chromatography revealed at least fifteen peaks, which were positive to the alkaline-hydrolysis test but were neither peptides nor glycosidic residues.

Similarly prepared Maillard ovalbumin (2x crystalline) tagged with either ¹⁴C-lysine or-tryptophan was also fed to rats to assess the fecal recovery. Feeding the Maillard
ovalbumin raised the recovery of radioactivity from 1 to 7% for the 'lysine' label, and from .2 to 3% for the 'tryptophan' label. Refeeding the 'lysine'-labeled extract increased the recovery to 15%.
INTRODUCTION

In digestibility and nutritional studies of proteins treated by the Maillard reaction (for a review see Carpenter and Booth, 1973), several authors have suggested that the binding of sugar residues makes certain regions of the protein undigestable, unabsorable and/or unavailable for animal growth (Clark and Tannenbaum, 1970; Valle-Riestra and Barnes, 1970; Sgarbieri et al., 1973). However feasible, this hypothesis has not been tested, or its importance evaluated as a possible mechanism for the loss of nutritive value of mildly brown proteins.

Two types of Maillard browning reactions should be distinguished according to the heating conditions. One that is carried out at mild, below denaturing, temperatures,¹ and another at high, processing or sterilizing temperatures. Under mild conditions, the reaction due to the presence of sugars, could be observed without the intervention of heat denaturation (insolubilization, condensation, oxidation). The duration of the treatment, of course, is an important factor.

The type of physiocochemical or conformational changes introduced to a protein within one month of mild heat treatment could be minimal, so that any digestive and

¹As the term shall be used throughout this report.
nutritive changes observed when exposed to reducing sugars can be mostly ascribed to the chemical addition of sugar moieties to the protein. Kline and Stewart (1948) reported no change in the solubility of egg albumin when stored with glucose at 25°C for up to forty-five days. The fluorescence and color development, however, indicated the progress of the Maillard reaction. Amaya, et al. (1974) reacted crystalline insulin with glucose at 37°C for two weeks, and observed that an average of 1.4 sugar residues per molecule of insulin had bound the polypeptide chains. Although the solubility had meanwhile increased about ten times, because of the dissociation of the hexamer into soluble dimers, the biological activity of two of the hormone’s functions had suffered little or no reduction. It was concluded that neither the mild heat treatment nor the modification by the 1-deoxy-2-ketosyl group had distorted the tertiary structure to any significant extent.

Besides the terminal amino groups of a protein, the only other amino groups likely to react with a reducing sugar is the ε-amino group of lysine (pK 10.51) and the guanidinium group of arginine (pK 12.48). Their high pK values raise the energies of activation making the reaction rate relatively low for the ε-amino and negligible for the guanidinium, compared to the α-amino group. (The guanidinium group of insulin is known not to react with glyoxal at pH 7; Nakaya, et al. 1967.) It would seem, therefore, that for mildly brown proteins, the entire mechanism of 'unavailable
peptides' would have to rest on either or both of the following assumptions:

(a) α-N-ketosylated polypeptides, which contain essential amino acids, must be excluded from uptake and surface hydrolysis in the mucosal cells (Matthews, 1971); (b) ε-N-substituted lysine must significantly delay endopeptidase action, hindering the release of neighboring residues.

Related to the first assumption, we have recently reported that the major product of the reaction of glycyl-L-leucine with glucose is not taken up by the small intestine of the rat; in fact it was observed that neither the whole Maillard dipeptide, nor any of its parts were able to cross the wall of the small intestine (Amaya et al., 1974). Regarding the second assumption, not much more is known either. Folk (1956) claimed that trypsin-mediated hydrolysis of a lysine-containing peptide was significantly lowered after allowing the ε-amino group to react with glucose. It is also known that ε-N-fructosyl-lysine can slowly diffuse through the intestine though it is hardly available for growth (Erbersdobler, 1973).

It would not be a difficult task to explain the loss of nutritive value of egg albumin after autoclaving for one hour in the presence of glucose, given that 70% of the 'lysine' is recovered in the feces (Valle-Riestra and Barnes, 1970). A different matter is to explain the 50% loss of protein efficiency ratio in egg albumin which was kept with
glucose at 37° for ten days. Under the latter conditions it is doubtful that any substantial amounts of lysine have yet reacted.

It was desirable in the present study to determine if fructosyl peptides, formed during the mild Maillard reaction of egg albumin, are retrievable from the feces of rats fed such protein. And if so, to assess the nutritional importance of the excreted residues.

**Feeding the Maillard system**

A homogeneous mixture of egg albumin (Nutritional Biochemicals Corporation, Cleveland, Ohio) was dissolved in water and 1.0% (v/v) was prepared and incubated for 60 min at 37° and 90° F. After brown, the protein was filtered and those containing 10% of either water were lyophilized. The recovery were made. Between the third and fourth stage, of feeding the diets in groups of five mice 5 in each, the mouse were collected and analyzed. All the techniques (N) were enzymatic method using as the reactant the protein to the preparations and checking of the diets were already described in previous research but not evaluation of the protein by this study at all.
MATERIALS AND METHODS

Radioactive ovalbumin

One laying hen was injected with either 200 u Ci of UL-\textsuperscript{14}C-L-lysine or 100 u Ci of 2 (ring)-\textsuperscript{14}C-DL-tryptophan (International Chemical and Nuclear Corporation, Irvine, California) in the wing vein avoiding any undue excitement of the bird. The first egg after the injection was collected and the albumin twice crystallized according to the method of Kekwick and Cannan (1936). The radioactivity recovered throughout crystallization and dialysis was 81.2% for the lysine-labeled and 86.1% for the tryptophan-labeled product.

Feeding the Maillard protein

A homogeneous mixture of egg albumin (Nutritional Biochemicals Corporation, Cleveland, Ohio) and glucose (3:2, w/w) was prepared and incubated for 30 days at 37° and 68% R.H. After brown, the protein was dialyzed and diets containing 10% of either untreated (U) or brown (M) protein were made. Between the third and fifth weeks of feeding the diets to groups of five growing rats, the feces were collected and analyzed. All the steps involved from the browning of the protein to the preparation and feeding of the diets were already described in detail in the nutritional evaluation of the proteins by Sgarbieri et al. (1973).
The radioactive and 'cold' ovalbumins were incorporated into diets as explained above and fed to pairs of male (Sprague-Dawley, Charles River Breeding Laboratories, Wilmington, Massachusetts) rats, weighing about 100 g each. The rats were trained for five days to eat two meals a day (30-45 min. long) of similar 'cold' egg albumin diet. Six hours after eating the radioactive meals the rats were placed in cylindrical turkey-wire restrainers to prevent coprophagy, and the feces were collected individually for the next 66 hours. For the refeeding experiment, two rats were preconditioned to the two-meal-a-day schedule with the following amino acid diet (Nutritional Biochemicals Corporation):

- Synthetic amino acid mixture 10%
- Sucrose 20%
- Cellulose 54%
- Jones-Foster salt mixture 5%
- Vitamin fortification mixture (see Sgarbieri et al., 1973) 1%
- Corn oil 10%

The water-soluble extracts of the feces containing the lysine-labeled material were added to the above diet and fed in 6-g meals. Coprophagy was prevented and the feces were collected daily for three days.

**Analysis of the feces**

The water-soluble fraction of the feces was extracted and passed through a polyacrylamide gel column using either
(i) .05 M NaCl, or (ii) .05 M ammonium acetate, or (iii) .2-.5 M pyridine acetate (pH 4.1).

(i) Amounts varying between .45 to .75 g of the lyophilized, ground material were extracted once with 6 ml of .05 M NaCl, the insoluble material sedimented at 12,000 xg for ten minutes, and the supernatant decanted through glass wool. The clear supernatant, which was of an intense brown color (for those feces proceeding from Maillard protein) was filtered through a Bio-Gel P-4 column (2.5 x 50 cm; 200-400 mesh, Bio-Rad Laboratories, Richmond, California) using .05 M NaCl as eluant. Other conditions were: Flow rate, 100 ml/hr; void volume, 240 ml; fraction size, 10 ml. The fractionated column was stored at either 4°C or -20°C if analyses were not immediately performed. The Bio-Gel column could be reused consecutively for two weeks, provided that it be washed for 8-10 hrs. with .05 M NaCl to remove a low-molecular weight fluorescent pigment which was present in all fecal preparations.

The following tests were performed on .2 - .5 ml of sample: Protein Lowry (Hartree, 1972), ninhydrin (Hirs, 1967), and ferricyanide (Borsook et al., 1955) for the typical ketosyl residue of glucose-generated Maillard compounds. The molecular weight of each peak was estimated, after concentration of the pooled fractions, in a Bio-Gel P-2 column (200-400 mesh; 1 x 50 cm; eluant, .05 M NaCl; void volume, 55 ml; flow rate 25 ml/hr.; fraction size, 6.5 ml). The markers were: serum albumin (BSA), vitamin B-12 and riboflavin.
Amino acid analysis was also performed on each one of the bands obtained from the NaCl/Bio-Gel patterns. For this, the pooled lyophilized fractions were efficiently desalted by extracting twice with 95% ethanol at room temperature, and then centrifuging at 10,000 x g for ten minutes. After evaporation of the 95% ethanol, the residue was hydrolyzed with redistilled 20% HCl for 22 hours. The HCl was removed by repeated evaporation at 45°, and the hydrolysates were chromatographed in a Technicon Auto Analyzer.

(ii) Ammonium acetate (.05 M, pH 5.4) was used in a separate trial to extract the soluble material and to elute a Bio-Gel P-4 column (already described). Fractions 32 through 42, beyond the void volume, which contained most of the brown, fluorescent band (700-1500 mol.-wt. region) were pooled and twice lyophilized. An aliquot of this product was directly analyzed for its amino acid content after HCl and alkaline hydrolysis (Spies and Chambers, 1949), and the rest was rechromatographed in a Dowex 50W X-4 column (90 x 1.6 cm; see Appendix B for gradient). Amino acid analyses were again performed on each of the peaks obtained from the Dowex column.

(iii) In addition to the above, the fecal material was also extracted and gel-filtered as indicated in (i), except that .2 M pyridine acetate (pH 4.1) was used instead of .05 M NaCl. The ninhydrin-positive bands were concentrated by evaporation, and subsequently lyophilized, followed by rechromatography in a Dowex column as described in (ii).
Each of the peaks of the cation exchange column was subject to the following test: amino acid analysis (Technicon Auto Analyzer), rapid alkaline hydrolysis (see Appendix B), the ketose ferricyanide reaction (Borsook et al., 1955), and the fluorescamine reaction for amines (Undenfriend et al., 1972; Fluram T.M., Hoffmann-LaRoche, Inc., Nutley, New Jersey).
### TABLE 4A

Accumulation of radioactivity in the feces of rats as a result of feeding Maillard ovalbumins labeled in either the lysyl or tryptophyl residues

| Type of Diet Adaptation | Type of Ovalbumin Fed (amount in g) | dpm Fed | dpm Recov. | Radioactivity Recovery |
|------------------------|------------------------------------|---------|------------|-----------------------|
|                        |                                     |         |            |                       |
| U                      | $^{14}$C-Lys-                       | 1292500 | 12202      | .94 ± 1.32            |
|                        | idem                               | (5)     | 1292500    | 1.13 ± .27            |
| M                      | $^{14}$C-Lys-                       | (8.6)   | 2223100    | 1.04 ± 1.06           |
|                        | idem                               | (5.4)   | 1395900    | 1.06 ± .03            |
| U                      | $^{14}$C-Lys-Maillard-              | (6.1)   | 3574250    | 6.70 ± 6.72           |
|                        | idem                               | (6.1)   | 3474250    | 6.73 ± 6.73           |
| U                      | $^{14}$C-Trp-                       | (5)     | 507660     | .20 ± .17             |
|                        | idem                               | (4.7)   | 472055     | .17 ± .05             |
| M                      | $^{14}$C-Trp-Maillard-              | (5.3)   | 171375     | 3.5 ± 3.25            |
|                        | idem                               | (7.2)   | 230956     | 3.0 ± 3.5             |

a. Radioactive ovalbumin was prepared by injecting hens with labeled amino acid, and then rice crystallizing with sodium sulfate. To make the Maillard ovalbumins, each protein was reacted with excess glucose in the 'dry state' at 37°C for 30 days.

b. Rats were individually trained to eat two meals a day of 'cold' ovalbumin, which was either unreacted (U) or Maillard (M) for the five days that preceded the experiment. During the test, the rats were coprogagy prevented. See experimental section for other details.
TABLE 4B

Further accumulation of radioactivity in the feces upon refeeding\textsuperscript{a} of the water-soluble fraction to two rats

| Type of Diet Adaptation | Type of Ovalbumin Fed (amount in g) | dpm Fed | dpm Recov. | Radioactivity Recovery % |
|-------------------------|-----------------------------------|---------|------------|------------------------|
| AA\textsuperscript{b}    | 14\textsuperscript{C}-Lys-Maillard- (6) | 139800  | 25014      | 17.9 \textsuperscript{c} |
| AA                      | Idem (6)                          | 99500   | 11412      | 11.5 \textsuperscript{c} |

\textsuperscript{a} The lyophilized, water-soluble material was incorporated to an amino acid diet, in which the starch was replaced by cellulose (see experimental section).

\textsuperscript{b} The rats were trained as in Table 4A except that a synthetic amino acid mixture was used instead of ovalbumin.
 Nitrogen distribution in the feces of rats fed either normal or Maillard egg albumin according to the watersolubility of the nitrogenous compounds

| Source and Fraction | N mg/g dry feces ± S.D. |
|---------------------|--------------------------|
| normal (2)b insoluble | 29.93 ± .81 |
| Maillard (2) insoluble | 35.33 ± .78 |
| normal (2) soluble | 6.77 ± .42 |
| Maillard (4) soluble | 31.86 ± 2.08 |

a. Samples of .2 to .6 g of dry, ground feces were extracted and sedimented three times in 10 ml of .05 M NaCl, the resulting fractions lyophilized and the N determined in a semi-nitrobokjeldahl apparatus. Values were corrected for NaCl and moisture content.

b. Number of replicates.
RESULTS

Feeding the Radioactive Ovalbumin

Six growing rats were fed a standard diet containing Maillard ovalbumin which was radioactively labeled in either the lysyl or the tryptophyl residues. Table 4A compared the fecal recovery of radioactivity from these rats with that of four rats that received unreacted ovalbumin. On the average, close to 90% of the radioactivity was found in the water-soluble fraction of the feces, and, about 7% in the low-molecular weight region. The storage-like browning produced a five-fold increase in the recovery of fecal radioactivity for the $^{14}$C-lysine ovalbumin. Browning of the $^{14}$C-tryptophan ovalbumin brought about an increase greater than ten-fold over the background of the unreacted ovalbumin (Table 4A).

When the water-soluble fraction of the feces from the $^{14}$C-lysine ovalbumin was refed, to two rats, the recovery of radioactivity was raised from 6.7 to 14.7%. The results of this experiment are recorded in Table 4B.

Analysis of the Water-Soluble Fraction

The following analysis was carried out on feces collected during the last two weeks of a five-week feeding trial, in which non-crystalline egg albumin was used instead of ovalbumin. Table 5 shows the distribution of nitrogen, according to its water (NaCl) solubility, in the feces,
Figure 7. Gel filtration pattern of the soluble fraction of feces of rats fed untreated (–○–) and Maillard (–x–) egg albumin. The extraction and elution was done with 0.05 M NaCl in a Bio-Gel P-4 column. When the Maillard albumin itself was extracted and eluted in the same manner, a different pattern (–++–) was obtained. The molecular weights estimated for bands I, II and III were 1300, 1000 and 800 respectively. The columns were developed with the ninhydrin reagent, and read between 570 and 640 nm. Other conditions for the column were: column size, 2.5 x 50 cm; fraction size, 10 ml; flow rate, 100 ml/hr.

Note that the commercial spray-dried egg albumin generates a good background of residues.
FIGURE 7

FRACTIONS

MINHYDRIN COLOR

I
II
III
TABLE 6

Molecular weights of the three bands with Maillard product characteristics isolated from the peptide region of fecal water extracts\textsuperscript{a}

| Compound | $\frac{Ve}{Vo} \pm \text{S.D.}\textsuperscript{b}$ | Molecular Weight |
|----------|---------------------------------|-----------------|
| Band I   | 1.330 ± .07                     | 1300            |
| Band II  | 1.812 ± .06                     | 1000            |
| Band III | 2.265 ± .103                    | 880             |
| Riboflavin| 4.916                           | 376.4           |
| B-12     | 1.383                           | 1300            |
| BSAC\textsuperscript{c} | 1.000 | excluded |

\textsuperscript{a} Maillard egg albumin was the protein fed. Corresponds to pattern in Figure 1.

\textsuperscript{b} Ve is elution volume. Vo is void volume. The column bed was Bio-Gel P-2 (200-400 mesh) and the eluant .05 M NaCl. The average of two trials is reported.

\textsuperscript{c} BSA is bovine serum albumin.
generated from both Maillard and unreacted egg albumin. It was apparent that the total nitrogen of the water-soluble fraction of the feces generated by Maillard egg albumin rose 470% while the insoluble nitrogen was only 16% above the control.

Extraction and gel-filtration of the water-soluble portions was accomplished by using three different solutions. (i) The polyacrylamide Bio-Gel filtration pattern shown in Figure 7 was obtained extracting with a .05 M NaCl solution the fecal material from both unreacted and Maillard protein sources. The column was then developed with the same solution. The higher ninhydrin color of the Maillard with respect to the normal pattern reflects the accumulation of nitrogen in the feces when the protein is brown. The same extraction and elution procedure was repeated directly on the Maillard egg albumin; no bands were detected in the peptide region. This pattern was quite reproducible for one preparation of feces, and was consistent with three totally independent preparations. It was noted, however, that the height of band III occasionally varied in proportion to the other two. It also must be noted that the 'unreacted' commercial egg albumin was not free from some accumulation.

The material present in these bands ranged between 800 and 1300 molecular weight (Table 6). The ketose/ferricyanide test was positive for the three bands, but most prominently so for band II (Figure 8). Amino acid
Figure 8. The material collected in the bands shown in Figure 1 had ketose character. The ninhydrin-positive profile (-o-) coincides with the alkaline ferricyanide (-α-) profile. The pattern shown is for Maillard-protein feces. Conditions for extraction and elution were given in Figure 7.
Amino acid composition of 'unavailable peptides' in the water-soluble fraction of the feces of rats fed commercial and Maillard egg albumin

| Amino acid HCl hydrolysate | Egg albumin Bio-Gel peaks (micromole/g) | Maillard egg albumin Bio-Gel peaks |
|-----------------------------|----------------------------------------|------------------------------------|
|                             | I | II | III |                             | I | II | III |
| Asp                         | 0.30 | 0.49 |     | 4.24 |
| Thr                         | 0.22 |     |     | 3.69 |
| Ser                         | 0.15 |     |     | 3.25 |
| Glu                         |     |     |     | 22.62 |
| Glc                         |     |     |     | 2.97 |
| Gly                         | 13.09 | 2.94 |     | 14.44 |
| Ala                         | 10.80 |     |     | 10.37 |
| Val                         | 3.99 |     | 1.21 | 2.25 |
| Met                         | 0.38 |     | 1.71 | 1.34 |
| Ile                         | 2.15 |     | 20.64 | 1.71 |
| Leu                         | 2.90 |     |     | 5.86 |
| Tyr                         |     |     |     | 2.53 |
| Phe                         |     |     | 0.38 | 1.32 |
| NH₃                         | 2.01 | 3.27 | 21.38 | 59.79 | 21.92 | 46.26 |
| Glc                        |     | 1.70 |     | 5.08 | 7.39 | 0.65 |
| Lys                         | 3.71 |     |     | 16.95 | 15.07 | 0.65 |
| His                         |     | 2.40 |     | 3.69 |
| Arg                         |     | 6.27 | 10.01 | 1.95 |
| Others                      |     |     |     | 1.51 |

a. Commercial egg albumin was the dialyzed, spray-dried egg white from Nutritional Biochemicals Corp. The Maillard type was the above protein browned for 30 days and then dialyzed. The diet was a standard formulation containing 10% of either protein.

b. Per gram of dry feces. Uncorrected for losses at the steps of extraction and pooling of the fractions.

c. Unidentified peaks in the chromatograms.
analysis was also performed on each band after HCl hydrolysis. Four major features stand out from the amino acid data in Table 7. (a) As a result of browning, the amino acid pattern became diversified; (b) most amino acids increased in concentration; (c) the proportion of basic amino acids and ammonia increased, and (d) the proportion of glycine was decreased by that of glutamic acid and isoleucine were notably increased. Another feature, which was not necessarily related to the degree of browning, was that acid hydrolysis of band III yielded almost exclusively NH₃. No tryptophan could be detected in the peptide region of the extracts.

Using this gel filtration system, the water-soluble extract of radioactive feces from Maillard ¹⁴C-Lys ovalbumin was eluted. Figure 9 indicated that about 80% of this radioactivity co-chromatographed with peaks I and II of a 'cold'-egg albumin fecal carrier.

(ii) The dried, ground feces were alternatively extracted and eluted with .05 M ammonium acetate. Although this system was capable of separating a diffuse band which appeared brown, uv-fluorescent, and low in molecular weight, it did not provide a reproducible ninhydrin profile after removal of excess ammonium ion. The 'band' thus prepared, however, was used to demonstrate the presence of several peptide-like bands as resolved by cation exchange. Figure 10 shows a typical ninhydrin profile on a Dowex 50W X-4 column. Table 8 gives the internal amino acid composition
Figure 9. Distribution of 'lysine' radioactivity in the feces of rats fed ovalbumin. About $10^5$ dpm were mixed with a brown extract as carrier. The gel filtration conditions were identical to those in Figure 7.

Code: Radioactivity (−x−); ninhydrin (−o−).
Figure 10. Comparison of the Dowex cation-exchange profiles of peptide-like compounds from the feces of rats fed Maillard (---) and untreated egg albumin (---). The feces were first extracted with .05 M NH$_4$AC, and then filtered in a Bio-Gel P-4 column with the same eluant. The amino acid analyses of these peaks appear in Table 8. Conditions for the Dowex 50W X-4 column are given in Appendix B. The input of the untreated was four times that of the Maillard material. Between fractions 140 and 160 there was a large band which was thought to be exchanged NH$_4$ ion.


**TABLE 8**

Internal amino acid composition (mole %) of five peaks from the Dowex 50W X-4 column in Figure 10

| Amino Acid | D-1 | D-2 | b | D-4 | D-5 | D-6 |
|------------|-----|-----|---|-----|-----|-----|
| Asp        | 70.9|     |   | 3.5 | 7.6 | 6.2 |
| Thr        | 14.8|     |   | 1.1 | 4.9 | 2.3 |
| Ser        | 2.2 |     |   | 2.3 | 7.0 | 5.9 |
| Glu        | 1.7 | 88.9|   | 1.6 | 13.6| 4.9 |
| Gly        | 2.5 | 2.4 |   | 1.8 | 9.2 | 9.0 |
| Ala        | .8  |     |   | 1.7 | 6.8 | 21.4|
| Val        | 1.6 |     |   | 84.4| 3.2 | 6.5 |
| Cys        | 2.7 | 6.7 |   | 14.1| 6.8 |   |
| Met        | .8  |     |   |     |     | 2.4 |
| Ile        |     |     |   |     |     | 11.0|
| Leu        |     |     |   |     |     | 15.8|
| Lys        | 1.8 | 1.9 |   | 3.3 | 12.0| 7.4 |
| His        |     |     |   |     |     |   |
| Arg        |     | .18.8| |     |     | 2.5 |

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*a. All peaks contained large amounts of ammonia which was not included in calculating the amino acid content.*

*b. Peak D-3 was lost accidentally.*
of each band after HCl hydrolysis.

(iii) In addition to the above separation procedures, the feces were also extracted and eluted through a Bio-Gel column with .2 – .5 M pyridine acetate (pH 4.1). The ninhydrin profile (Figure 11) was clearly different from that obtained in (i).

Moreover, when bands II and III were re-chromatographed in a Dowex column (Figures 12A, 12B, the ninhydrin profile differed from that of the ammonium acetate extraction. After HCl hydrolysis, each peak in Figure 12A, 12B was found to yield about 100% ammonia. A negative reaction of each one of these peaks to the Elson-Morgan reagent eliminated the possibility of dealing with undigested glycosidic moieties of glycoproteins.

All the Dowex peaks increased their ninhydrin color absorbance by an average factor of 4.4 after alkaline hydrolysis. This was consistent with the assumed polymeric nature of the compounds. Fluorescamine, a compound that reacts with primary amines to form strongly fluorescent chromophores, reacted rather weakly with all the peaks tested but the fluorescence increased slightly in the direction of basicity (Table 9).
Figure 11. Gel filtration pattern of pyridine-acetate soluble extracts of the feces of rats fed Maillard (−○−) or untreated (−×−) egg albumin. The patterns were obtained from a Bio-Gel P-4 column, like that described in Figure 7, except that it was eluted with .5 M pyridine acetate. The color from equal inputs was developed with ninhydrin.
FIGURE 11

FRACTIONS

NINHYDRIN O.D.

1.4

1.2

1.0

0.8

0.6

0.4

10 20 30 40 50
Figure 12A. Cation exchange/ninhydrin profile of both Maillard (-○-) and non-Maillard (···) type of feces, as obtained from the pyridine-acetate preparation band II (Figure 11). The ketose ferricyanide reaction for the Maillard profile (~x~) is included. Other tests done on this column are summarized in Table 9. The Dowex column and the gradient are described in Appendix B.

Figure 12B. Cation exchange/ninhydrin profile of the Maillard type of feces (-○-) from the pyridine acetate preparation band III (Figure 11). The ketose ferricyanide reaction (~x~) is also shown. The Dowex column and the gradient were identical to those in Figure 12A.
Figure 12B
TABLE 9

Summary of the chemical tests performed on the Dowex fractions of the Bio-Gel/pyridine acetate peaks II and III whose profile is shown in Figure 12A,B.

| Reaction | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ferricyanide<sup>b</sup> | + | + | + | - | - | - | + | + | + | + | + | - | - | - |
| Alk. Hydrolysis<sup>c</sup> | 6.8 | 5.7 | 2.9 | 5.2 | 4.0 | 5.2 | 5.6 | 4.1 | 3.5 | 3.2 | 2.5 | 2.5 | - | - |
| Elson-Morgan<sup>d</sup> | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fluorescamine<sup>e</sup> | 1.2 | 0.9 | 1.4 | 1.6 | 1.2 | 3.0 | 1.6 | 1.9 | 2.1 | 2.5 | - | - | - | - |
| HCl Hydrolysis<sup>f</sup> | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

II Series

III Series

| Reaction | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|----------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ferricyanide<sup>b</sup> | + | + | + | - | - | - | + | + | + | + | + | - | - | - |
| Alk. Hydrolysis<sup>c</sup> | 6.8 | 5.7 | 2.9 | 5.2 | 4.0 | 5.2 | 5.6 | 4.1 | 3.5 | 3.2 | 2.5 | 2.5 | - | - |
| Elson-Morgan<sup>d</sup> | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fluorescamine<sup>e</sup> | 1.2 | 0.9 | 1.4 | 1.6 | 1.2 | 3.0 | 1.6 | 1.9 | 2.1 | 2.5 | - | - | - | - |
| HCl Hydrolysis<sup>f</sup> | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

(100%)

a. Dowex 50W X-4 separation of bands II, III from the Bio-Gel/P-4/pyridine acetate system. Feces were from the rats fed Maillard egg albumin.

b. If ketoses as in the Amadori compound—are present, the ferricyanide ion is reduced and forms Prussian blue (660 nm).

c. Samples of .5 ml are hydrolyzed in 1 ml of 2.5 N KOH in the autoclave for 50 min., then neutralized with .6 ml of 30% AcOH, and the ninhydrin test is compared against the unhydrolyzed sample.

d. Is a hexosamine determination with the Earlich's reagent (Johnson, 1971).

e. Is a fluorescent reaction for the determination of primary amines. It was carried out in borate buffer pH 8.0 (Undenfriend et al., 1972). Values are ratios of fluorescence over ninhydrin color. The value for n-leucine was 2.2.

f. Samples were concentrated enough to obviate removal of salts. NH₃ was the only peak observed for the II series. The III series were (%): A: (NH₃, 50.9; Glu, 49.1). B: (NH₃, 60.6; Ala, 26.3; Glu, 6.9; Gly, 6.1). C: (NH₃, 100).
DISCUSSION

From the feeding and refeeding experiments it was apparent that mild Maillard browning of ovalbumin made certain portions of the polypeptide chain, bearing a $^{14}$C-tagging essential amino acid, from four to ten times less likely to be absorbed by the intestine of the rat (Table 4). The concentration of lysine and arginine were indisputably elevated in the fecal water-soluble fraction after browning (Table 7). It was also observed that, besides aspartic and glutamic acids, the neutral amino acids glycine, alanine, valine, isoleucine and leucine were found in high proportions in some of the isolated Dowex peaks (Table 8). Since neutral amino acids participate in the Maillard reaction only if they are N-terminal residues, it would not be surprising if a major egg protein, for instance, has either glycine or leucine or isoleucine as its N-terminal residue (Table 8, peak D-6). It has been shown by Amaya et al. (1974) that in the small intestine of the rat no leucine is absorbed in vivo from the Maillard dipeptide glycyl-L-leucine.

Although the level of fecal excretion was below 10% of the ingested radioactivity, this loss was considered important because (a) the radioactivity was concentrated between the 1000 and 1300 molecular weight fragment (Figure 9); (b) the relative nutritive value associated with such
protein was estimated to be 7% (Sgarbieri et al., 1973; (c) the absence of radioactivity from the 800-molecular weight band warranted investigation of the nature of such band and its role in the loss of nutritive value induced by mild Maillard browning.

Aqueous solutions of sodium chloride and ammonium acetate extracted compounds which, upon acid hydrolysis, yielded various amino acids plus ammonia or ammonia alone. Meanwhile, pyridine acetate appeared to selectively extract the ammonia-yielding compounds characteristic of the low-molecular weight band. Bands III (Figure 7), II and III (Figure 11) were major contributors to the total soluble nitrogen of the feces. It was shown by alkaline hydrolysis that this nitrogen was not ionically bound NH$_4^+$, but rather in the form of primary or secondary non-peptide amines. Their excellent solubility in ethanol and pyridine also suggested the compounds were not peptides.

One remote source of non-protein nitrogen with Maillard characteristics in the feces was the possibly undigested glycosidic moieties of the albumin's glycoproteins. This possibility was discarded after the negative results of the Elson-Morgan reaction for hexosamines. Another source considered was that of bacterial or intestinal origin, but this was unlikely given the molecular weight range and the very Maillard characteristics of the band.
So far as the author is aware, the presence of polyamines in egg white proteins has not been reported. Nevertheless, it is possible that such important growth factors (Russell, 1973) are bound to multiple phosphate groups of ovalbumin, phosvitin, etc. -- just as they are found in nucleic acids. Spermine, the largest common polyamine, has a molecular weight of 188. A most likely Maillard product, the tetra-substituted derivative, would have a molecular weight of 828.

Furthermore, if polyamines are present in egg white and milk proteins, and they readily react with reducing sugars, they may provide answers to some of the remaining questions about the nutritional effect of the Maillard reaction.

Our data suggest that mild Maillard browning is not sufficient treatment to produce high lysine losses (Table 4) as is known to occur with autoclaved Maillard proteins (Valle-Riestra and Barnes, 1970). This contention is in agreement with investigations on the glucose-to-lysine binding in proteins (Schwartz and Lea, 1950; Amaya et al., 1974), and also with the higher energy of activation required for the nucleophylic attack on the carbonyl carbon of the sugar by the ε-amino group.

In contrast with the apparently little amino acid damage, Tanaka et al. (1974) have reported that as much as 50% of the nutritive value is lost after ten days of browning. These authors also suggested that growing rats could be more sensitive to the inferior quality of Maillard protein
than are adult rats. Sgarbieri et al. (1973) came to con-
clude that even a thorough amino acid supplementation did
not completely restore the lost nutritive value of a
mildly browned Maillard protein.

Maillard proteins reacted at high temperatures should,
therefore, be distinguished from those reacted in mild con-
ditions. In the first case, recoveries of up to 70% of the
ingested lysine radioactivity have been reported (Valle-
Riestra and Barnes, 1970), whereas less than 7% is recovered
in the second case despite the low nutritive value of the
protein. Whether the mild treatment is to result in an in-
creased absorption of still unutilizable lysine (because of
the less cross linking and higher distibility) is not known.
For certain, the low $^{14}C$ radioactivity recovered in the
feces when labeled ovalbumin was fed does not seem to be a
nutritionally important loss. It must be emphasized that,
to the present, no conclusive evidence has been found on
the possible toxicity of mildly browned Maillard proteins.

The recent finding that free radical products can
form early in sugar-amine reactions (Namiki et al., 1973)
should be given ample consideration, not only from the
chemical but also from the nutritional standpoint. Free
radicals formed at 100$^\circ$ could add to and cross link amino
acid residues in a protein thus contributing to its insolu-
bility and undigestibility. It would be of interest to study
the importance and stability of the Maillard reaction free
radicals at mild temperatures.
In summary, evidence has been presented for the accumulation of (a) oligopeptide and (b) non-peptide nitrogen in the feces fed mildly browned egg albumin. Both types of residues exhibited the characteristics of Maillard products. The Maillard oligopeptides, three to five residues long, could have escaped absorption and surface hydrolysis in the intestine because of the lack of free $\alpha$-amino groups. From crystalline ovalbumin only 6% $^{14}$C-lysine radioactivity was recovered associated with the small peptides, a loss considered much too small compared to the loss of nutritive value of the Maillard protein. A justification has been presented for the investigation of the possible presence of polyamines in egg albumin and their destruction by reducing sugars as an alternative or complement to the theory of Maillard-induced toxicity.
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APPENDIX A

THE MAILLARD REACTION

As defined by the Merck Index, the Maillard or "browning" reaction is "the reaction of reducing sugars with amino acids leading to the evolution of CO₂ and the development of an intense brown color. The reaction is complex and involves the formation of numerous compounds; among those that have been identified are furfural derivatives, N-glycosides and reductones."

The Chemistry of the Maillard Reaction

Numerous reviews on the chemistry of the Maillard reaction exist but must add little to that of Hodge (1953), which will be the only one I refer to. Figure 1 is a scheme of the early steps of the reaction between a primary amino (an amino acid or a protein, etc.) and a reducing sugar such as glucose. Except at low pH, when the amino nitrogen is highly protonated, a glycosyl-amine adduct is formed which reversibly dehydrates giving rise to Schiff's bases. Isomerization of the double bond can yield a 1,2-eneaminol and a 2-keto compound by an Amadori-type of rearrangement. These ketosylamine compounds are somewhat stable and can be isolated often in white crystals, but they decompose if exposed to enough moisture and heat to form tar-like syrups. The most stable of these products is the one formed with the
FIGURE 13

INITIATION OF THE MAILLARD REACTION. IF THE KETO (AMADORI) COMPOUND IS NOT FORMED,
THERE IS NO MAILLARD BROWNING.
\(\varepsilon\)-amino group of lysine, which can stand temperatures of up to 100° (Finot and Mauron, 1969). That the Amadori product is an intermediate in the Maillard reaction, regardless of the conditions was suggested by Hodge.

Starting with the Amadori compound, the reaction can continue forming literally a myriad of products (Figures 13, 14). There is an intermediate stage during which the sugar moiety dehydrates to form diketo, triketo, and polyketo compounds called reductones, if the conditions are not acidic -- like in the dry state. Under acidic conditions, furfural derivatives are formed. In this stage there is also sugar fission which gives rise to familiar volatile products. The mechanism for fission appears to be the reverse of aldol condensation (dealdolization).

Another reaction that has been observed in the intermediate stage of browning is the Strecker degradation. In this degradation, an \(\alpha\)-amino acid decarboxylates if heated in the presence of certain polycarbonyl compounds to give an aldehyde containing one carbon less. The nitrogen atom is transferred to the keto compound -- just like in the ninhydrin reaction -- although there is no production of color. In fact, it is believed that the Strecker degradation accounts for about 80% of the \(\text{CO}_2\) formed and that it slows down the development of brown color.

In the third and final stage, the intermediates polymerize by aldol condensation, aldehyde-amine polymerization
and heterocyclization. The final product, called melanoidins, are fluorescent, unsaturated compounds that exhibit a brown color without an absorption maximum in the visible range.

Reevaluation of the mechanism for the reaction may be needed in the near future due to the recent detection of free radicals even at the initiation steps. Nakimi et al. (1973) suggested that an early amino-sugar intermediate is a stable free radical species in the reaction of D-arabinose and \( \beta \)-alanine.
FIGURE 14

SIMPLIFIED SCHEME SHOWING THE
THREE STAGES OF THE MAILLARD REACTION
A BRIEF HISTORICAL ACCOUNT

The Maillard reaction was regarded as little more than a chemical curiosity for quite some time after its discovery. During the two-and-one-half decades that followed L.C. Maillard's report in 1912 on the dramatic reaction that could occur between amino acids and glucose, no serious consideration was given to the finding by any field of science. It was not until the middle of the 1940's when the need arose to ship large quantities of dehydrated foods for troops stationed overseas that research on the reaction actually began to flourish. It is somewhat surprising to note that, even at that point, all interest in the reaction was triggered by the food processor's concern over the development of undesirable flavors and the low acceptance by soldiers of products such as dehydrated eggs.

Although the English workers, K.M. Henry, S.K. Kon and A.R. Patton, reported in 1948 that drastic losses in the nutritive value of milk could take place during the process of drying, the nutritional implications of the reaction were to remain forgotten until the middle or late 1960's. Meanwhile, the American workers, R.W. Kline and G.F. Stuart, were already publishing their observations on the physical-chemical changes induced by the reaction on dried egg albumin. By 1953, a good deal of knowledge about the reaction of proteins had been added through the physical-chemical studies on dry casein by C.H. Lea and R.S. Hannan in England.
Although the British investigators adopted casein, and the American counterparts egg albumin as the protein "model system," it soon became obvious that despite their practical relevance, such proteins were highly undefined and complex chemical entities. In fact, proteins were not the amines of choice in answering some of the basic questions about the chemistry of the reaction.

The chemical aspects, therefore, did not begin to unravel until small-molecule model systems were used; glycine and glucose, the mixture used by Maillard thirty-eight years before provided an excellent system for the chemist. Crucial were the contributions of C.O. Chichester, F.H. Stadtman and G. MacKinney who studied the products and the kinetics of the reaction using the then novel $^{14}$C-labeled reactants. Other names associated with the kinetics studies of the early fifties were G. Haugaard, L. Tummerman and H. Silvestri. As the research reports kept coming in at an exponential rate, the complexity of the reaction was quickly unveiled. Important were the work of M.L. Wolfrom in the U.S., and of C. Enders in Germany. Wolfrom proposed the involvement of the $\alpha,\beta$-unsaturation of the sugar in the formation of reductones, and pioneered the research on the nature of the sugar-amine melanoidins. Enders' contribution also was on the formation of melanoidins. It was also around that time when A. Gottschalk and J.E. Hodge were independently proving the significance of the Amadori compound in the Maillard reaction. All these studies culminated in what is known to be the most significant theoretical contribution to the chemistry of the reaction.
reaction; a unifying review article by J. Hodge in 1953. Far from being an anticlimax, H.S. Olcott reported that a simple fermentation process could be used to remove the reducing sugars present in eggs and other products, thereby providing a solution to the practical problem.

In looking through the literature, one gets the impression that a highly stimulating atmosphere existed at the time for the researchers working on the Maillard reaction. Unfortunately, this spirit practically died towards the beginning of the 1960's, partly because of the complexity of the reaction and partly because of priorities. Research on the chemistry of the reaction continued but at a slower pace and was maintained by the undying interest that has existed in the flavors, aromas and color of the reaction. This area of the reaction has been thoroughly reviewed by T.M. Reynolds in Australia (Reynolds, 1969).

A couple of misconceptions remained from all that work in the decade of the 1950's. The idea was put forth by Lea and Hannan (1950) that, even the peptide nitrogen of a protein is reactive towards the carbonyl carbon of a reducing sugar. This concept was neither proved nor disproved. It is now known that the reactivity of the peptide nitrogen is negligible given that its lone pair of electrons participate in the resonant structure of the peptide bond, and are not readily available for a nucleophilic attack. Such an idea, however, must have mislead Horn et al. (1963), who concluded that all the peptide nitrogen atoms of a protein must react with the carbohydrate.

Another idea, which was more like a passing fancy, was
based on the claim that ketose-amino acid (Amadori) compounds could stimulate the synthesis of protein in vitro (Borsook et al., 1955). Since this research was not followed up, and recent in vivo nutritional experiments attest to the contrary, the report is no longer considered of biochemical significance. These authors, however, did start a new age of research in the Maillard reaction; namely, the biochemical nutrition.

It should be added at this point that somewhat of a controversy has existed since 1966 about the production of toxic compounds by the Maillard reaction. Although volatile byproducts, such as furfuraldehyde derivatives are recognized as toxic, no deleterious effects have been found for other products of the reaction of food constituents. J. Adrian in France has been the major proponent of this hypothesis, but the properties of the premelanoidins he terms now as "anti-nutritional" instead of toxic (Adrian, 1974).

Since the second half of the 1960's a number of researchers in Germany, England, France, and in the United States, have been looking at the biochemical and physiological effects of the ketosyl-amino compounds. H. Ebersdobler's Laboratory in Germany has been testing fructoselysine in rats and microorganisms. Similar work is being carried out in France by P.A. Finot and J. Mauron. K.J. Carpenter's laboratory in England has been concerned in bringing together the chemical and biological methods to assess the loss of lysine during the Maillard reaction. In the United States there are a number of researchers doing part-time work in the field. Like their colleagues in
Europe, the American workers have been employing a combination of nutritional, biochemical and chemical approaches to further the understanding of the reaction. Among such researchers are R.H. Barnes (Cornell University), S. Tannenbaum (M.I.T.), A.E. Harper (University of Wisconsin), and C.O. Chichester (at this University).

Today, research on the Maillard reaction is in good standing but the emphasis is on its applications to the technology of foods, drugs, etc. Although for years the food processor has been removing reducing sugars from dried eggs or dried milk, and meats are only mildly susceptible to the Maillard reaction, there is a need for maximizing the efficiency of foods. With the trend for tapping more novel foods, usually of vegetable origin, more extensive processing is required, and the quality of the food depends on its susceptibility to heat. The current interest in minimizing the damaging effect to proteins and vitamins by the Maillard reaction, is coupled with a need to learn about any toxic properties that the Maillard products may have on animals.
APPENDIX B

AN IN VIVO ABSORPTION TECHNIQUE BY CANNULATION OF THE RAT

Introduction

This procedure allows the researcher to use every animal as its own control, with the use of a minimum amount of material for the biochemical study. When used with dexterity and under semi-aspectic conditions the animal can last for up to three days after the operation providing an excellent tool for some prolonged experiments. Up to 4 ml of blood were sometimes withdrawn from a 180 g rat without any physical detriment or change in the hematocrit. The whole operation should take from 1 to 2 hours, and the experiment can be conducted beginning one hour after the animal regains consciousness. A word of warning should be added about the use of the Eastman 910 glue. This glue has been banned from commercial use by the USFDA because of its dangerous effects when applied topically or swallowed by accident.

Making of the Cannulae

A stretch 10 m long of polyethylene tubing PE-50 (Clay Adams Intramedic polyethylene tubing PE 50 Cat #13-9125, Ace Scientific Supply Company, 1420 East Linden Avenue, Linden, New Jersey 07036), is flushed with 2% NaOH,
rinsed, siliconized (Silidad, Clay-Adams) rinsed with distilled water again, then dried overnight and cut in 30-cm segments. An 80-degree hook is made in each segment, at about 1.5-2 cm from one end, by inserting the segment in a glass tubing mold and dipping it first in boiling water and then in cool water. The cannulae are readied a day before use by filling them with heparin (1000 u/ml), occluding the straight end with a headless steel pin and putting a thin latex (like from latex or plastic gloves) collar, about 4 mm in diameter, through each cannula. Care should be taken that the heparin is prewarmed before filling the cannula so that no air bubbles form inside. The cannulae could be kept at room temperature for a couple of days. If nus.t develops near the pin, the tip could be cut and a new pin employed immediately after cannulation.

The Rats

Female Sprague-Dawley rats weighing 150-175 g are adapted for 3 or more days. According to Dr. Gallo-Torres, female rats are able to tolerate post-operative pain better than male rats. Also, the age of the rat is somewhat critical. The absorption pattern of rats weighing near or above 300 g show slow absorption. The operation of rats of about 300 g or older may present complications due to the increased susceptibility of the vein tissue to rip or tear.

After the adaptation period, the rats are fasted for 15 hours before the operation and the water is replaced with
saline (.85% NaCl). With fasting, peptide or protein absorption is enhanced, and the animals have better protection against death from anesthesia. Since on-and-off application of the Penthrane beaker will be necessary throughout the operation, it may be convenient to rewet the gauze with Penthrane as reflexes reappear in the animal despite sustained application of the beaker to the nozzle. The beaker should be removed as soon as the reflexes disappear. The number of rats prepared depends upon the number of cage retainers available and also your ability in performing the operation.

**The Cannulation**

The support of an assistant is recommended throughout the operation. The rat is anesthetized with four milliliters of Penthrane (#6864 Cornell Surgical Co., 224 61st Street, West New York, New Jersey 07093; desirable over ether because of its acceptance by the rat and humans) soaking a piece of gauze in a 150 ml beaker. With the aid of an operating table, the skin and the abdominal muscle are separately cut by the mid-line incision (strong dissecting scissors are recommended unless professional skill has been acquired with the scalpel). Taking care that the diaphragm is untouched, the proximal end of the incision should just begin to show the tip of the sternum when the skin and muscle are held up by the retracting hooks of the operating table (Figure 15).
The intestine is gently withdrawn to the left side and wrapped with a piece of gauze soaked and squeezed in saline at 40°C. The ensuing steps are as follows:

1. With a 20 G needle puncture the upper part of the duodenum about 1 cm below the stomach. Avoid any capillaries. Introduce the bent tip of a cannula (unsiliconized, unheparinized, precurved 12" tubing) and tie with black braided silk (Deknatel black braided silk/cardiovascular 1/2 circle tape, Ace Scientific Supply Company, Inc., 1420 East Linden Avenue, Linden, New Jersey 07036). A touch of alkyl cyanoacrylate glue (Eastman 910 adhesive, Cadillac Plastic & Chemical Company, 1761 Edgar Road, Linden, New Jersey 07036) between the tubing and the duodenum will seal any leaks in 15 seconds. The glue is best applied with presealed glass capillaries. If too much glue is used, the tissue will contract and get damaged.

2. Insert half way a 17 G hypodermic needle from the outside of the abdominal wall to meet the open end of the cannula so it can be drawn out as shown in Figure 15.

3. Holding the anterior lobe of the liver up and under the chest with a small piece of gauze (previously wetted in warm saline), expose the hepatic portal vein (Figure 15) so that it can stay flat and extended in the center of the abdominal cavity.

4. With a small pair of forceps (Milter, MX 18-780 eye dressing forceps, 4" straight, serrated delicate, Roboz
Figure 15
Cannulation of Duodenum and Portal Vein

L = Liver
K = Kidney
D = Duodenum
Surgical Instrument Company, 810 18th Street, N.W., Washington, D.C. 20006) hold a heparinized cannula and cut a bevel as shown in Figure 16b (use sharp curved scissors; eye scissors 4-1/2" Miltex MX 18-1434, Roboz Surgical Instrument Company, 810 18th Street, N.W., Washington, D.C. 20006). The shape and position of the bevel could be critical at the time of insertion and when collecting the blood. The bevel should be at an angle of 45°, the end should be sharp, and it should be facing you when the cannula lies flat on the table. Make sure the cannula remains completely filled with heparin.

5. While the right index or middle finger gently presses the vein down, and the cannula is held with the straight forceps, one plows over the vein making a groove at the center (Figure 16c, 16d) until piercing is accomplished just above the pyloric vein. Insertion is completed, first making sure that the tip of the cannula is inside and not engaged in piercing the other wall of the vein, and then following the tip until it has just passed the confluence with the splenic vein. If the tip goes a few millimeters below that point, there is danger of stopping blood flow through the cannula after surgery. This step is usually quite bloodless.

6. A touch of glue is applied to temporarily affix the cannula and the assistant can test the blood flow by releasing the occluding pin, with extreme care. Blood should flow continuously to at least halfway down the cannula to assure there is no impediment. The assistant can immediately return the blood with a syringe (23 G) filled with heparin, clamp it with a hemostat, and replace the pin. If
ACTUAL POINT OF INSERTION - BEVEL SHOULD LOOK UPWARD

HOLDING AND CUTTING BEVEL

PLowing down the center of the portal vein

COLLAR LIVER

PYLORIC

SPLENIC

ONE COULD SEE THE TIP OF THE CANNULA JUST BELOW THE SPLenic VEIN

APPLY A LIGHT TOUCH OF ADHESIVE NEAR THE COLLAR HOLE, NOT UNDER

FIGURE 16
there is no flow, the most common reason is occlusion of the bevel by the vein wall. Therefore, the cannula should be gently manipulated at the point of insertion while the other end is free until flow is evident. Care should be exercised when returning the blood so that no excess heparin is injected and no excess blood remains in the cannula; the blood meniscus should be returned judiciously until it has reached the beveled end.

7. Without disturbing the cemented cannula, the collar is slid down with the curved forceps (eye dressing forceps, half curved Miltex MX 18 782) until it reaches the portal vein, a minute drop of glue is applied to the top of the collar, and allowed to diffuse towards the tissue through the hole and the plastic. Hold down with forceps and wait for fifteen seconds. Then, while the assistant steadily holds the cannula about 2 cm away from the insertion point with the forceps, the abdominal wall is pierced with the 17 G needle as indicated in Figure 15, and the cannula drawn to the outside. Since one of the liver lobes lies under the upper part of the cannula, a touch or two of glue in this area will help to keep the cannula in place.

8. After removing the piece of gauze from under the chest, the intestine is gently replaced inside the cavity, the abdominal muscle layer sewed up with needle and thread, and the skin closed with autoclips (Clay Adams, 9 mm).

9. The animal is then secured in a restraining cage where the experiment is initiated one hour after it has
regained consciousness. Acceptance of water or saline water is considered a sign of good physical condition. The duodenal cannula should be occluded with a pin or a hemostat immediately after infusion of the substrate.
This modification involves the same basic steps already described except that a syringe is used instead of a cannula, and the experiment is conducted in the unclosed animal under Penthrane anesthesia. Since the operation can be shortened to about fifteen minutes prior to the duodenal infusion, this procedure is recommended for absorption studies of no more than one and one-half hours long.

Procedure

The rat is prepared in the same manner as indicated above up until the exposure of the abdominal cavity by the mid-line incision. A number of disposable tuberculin (1 cc) syringes with heparinized 23 G needles, heparinized small centrifuge tubes, and an extra supply of 23 G needles should be at hand. The remaining steps are as follows:

1. Gently take out the whole intestine to the left side and wrap it with a gauze pad (soaked and squeezed in saline at 40°C).

2. Fit a small piece of salinized gauze under the chest holding the major lobe of the liver out of the way so that the portal vein is visible.

3. With a 23 G needle (unheparinized) infuse the substrate into the duodenum at about 2 cm below the pyloric valve of the stomach.
4. Withdraw the syringe and promptly seal the puncture with a touch of Eastman 910 adhesive.

5. Gyrate the operating board $180^\circ$ on the table so that the rat's head is towards the operator.

6. At the preselected time for the first collection of blood (or a couple of minutes before) pierce the portal vein just proximal to the pyloric vein confluence with a heparinized 23 G needle and syringe.

7. Very carefully, drive the tip of the needle to a point inside the vein just distal to the splenic vein, and start withdrawing the necessary volume of blood. Since fresh needles could be too sharp and produce multiple punctures, the tip of the needle can be slightly dulled by tapping it against a hard surface.

8. The syringe is not withdrawn until it is time for the next collection of blood. Then, another syringe (with heparinized, predulled needle) is introduced through the same opening of the vein when the bevel (looking upwards) of the first needle is being withdrawn.

9. Steps 7 and 8 are then sequentially repeated for as many times as required without any significant loss of blood.

Attention should be called to the on-and-off application of the beaker with Penthrane. Although one person alone can carry out all the steps with no difficulty, care must be taken that the amount of anesthesia is always sufficient so that the animal cannot recover to the level of involuntary movements.
SPECIAL GRADIENT FOR AMINO ACID ANALYZER

When basic, small molecules such as L-lysinyl-L-tryptophan, or its Maillard derivatives are chromatographed in the amino acid analyzer, it is necessary to either prolong the elution time considerably -- which is economically impractical -- or increase both the pH and the ionic-strength gradients rather sharply.

The gradient employed was as follows

| Chamber | pH5 Buffer (ml) | .5 N NaOH | 1.2 N NaCl |
|---------|----------------|-----------|------------|
| 1       | 45 + 5 MeOH    | --        |            |
| 2       | 47 + 2 MeOH    | --        |            |
| 3       | 50             | --        |            |
| 4       | 50             | --        |            |
| 5       | 50             | --        |            |
| 6       | 40             | 10        |            |
| 7       | 25             | 25        |            |
| 8       | 25             | 25        |            |

The instrument used was the single-column (.6 x 140 cm; chromobeads "B" resin) semi-automatic Technicon Auto Analyzer (Technicon Company, Tarrytown, New York). This instrument has a constant flow rate of 30 ml/hr, and operates with the column at 60°C. The column is pre-equilibrated with citrate buffer pH for 3.8 to 1.5 hours. For the preparation of the citrate buffers, the Technicon directions were followed.
Gradient for Fecal Peptides. This gradient was devised to separate a number of fecal peptides using a Pharmacia 1.6 x 90 cm column (Pharmacia, Piscataway, New Jersey) at room temperature. The bed gel used was the Dowex 50W X-4 resin, which was extensively cleaned before use. The cleaning of the resin started by washing and decanting twice in one volume of 2N NaOH, followed by several rinses with H₂O, and finally washing twice in one volume of 2N HCl. After extensive rinsing with H₂O, the gel was equilibrated with starting buffer.

Buffer Solutions:

Buffer A:

14.71 g Na₃ citrate 2H₂O (.05M)
900 ml H₂O
Titrate with HCl to pH 2.5; make 1000 ml

Buffer B:

same as A, except pH 3.5

Buffer C:

14.71 g Na₃ citrate 2H₂O (.05M)
900 ml H₂O
35.07 g NaC L (.6M)
Titrate with HCl to pH 5.5; make 1000 ml

The Gradient

| Chamber no.: | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|--------------|---|---|---|---|---|---|---|---|---|
| Buffer:      | A | B | B | B | B | C | C | C | C |
| Volume:      | 75| 75| 75| 75| 75| 75| 75| 75| 75|
| Flow rate:   | 50 ml/hr  | Fraction volume: | 4 ml. |
THE ANALYSIS OF TRYPTOPHAN

**Introduction.** The determination of tryptophan in proteins has been the subject of controversy for many years because of its susceptibility to protonation and ring hydrolysis in strong HCl or because of its interference with neighboring residues in a protein. I have observed that acid hydrolysis of polytryptophan, either alone or in the presence of fructose, yields almost quantitative amounts of a compound that chromatographs in the position of tryptophan in the amino acid analyzer. Washington Tarqui of University Catolica in Chile, however, says that rats fed acid-hydrolyzed tryptophan do not grow. Alkaline or enzymatic hydrolysis of the protein, followed by colorimetric or chromatographic analysis constitutes, therefore, the most accepted methods of tryptophan determination in foods.

Spectrophotometric methods based on the UV spectrum of the indole group are not reliable because of interference from tyrosine or because of the insolubility of certain proteins, especially from foodstuffs. Recently, B. Holmquist from Harvard reported the rapid, accurate use of magnetic circular dichroism (MCD) in unhydrolyzed proteins. This method remains to be tested with food proteins but seems promising.

For free tryptophan, the best method I have used is that of Denkla and Dewey (1967) because of its high sensitivity and lack of interference, even from protein-bound
tryptophan. This method requires, therefore, the free α-amino group of the amino acid to form a fluorescent ad-duct (merhamman). The detailed procedure, as spelled out by John Fernstrom of MIT is as follows:

**Blood Tryptophan**

**Reagents:**

- **TCA/FeCl₃**: 8.1 g of FeCl₃·6H₂O in 100 ml of 10% TCA (prepare exactly as said).
- **HCHO**: 1.0 ml of 37% Formaldehyde + 19.5 ml of distilled H₂O.
- **Stock Tryptophan Standard**: 5.0 mg of L-tryptophan dissolved in 100 ml of 0.1 N NH₄OH.
- **Working Tryptophan Standard**: 5 mg/100 ml = 5 ug/100 ul.
- **10% TCA**: 10 g TCA in 100 ml distilled H₂O.

**Procedure for Samples:**

1. Place 1.8 ml of TCA/FeCl₃ into siliconized pyrex Sorvall centrifuge tubes (the ones that fit the SM-24 head).
2. Add 25 ul aliquots of serum or plasma.
3. **Vortex immediately.**
4. Centrifuge at 13,000 rpm for 10 minutes.
5. Decant supernatants into tubes that can be capped (if not pyrex, then do not cool in water. Just air is fine).
6. Add 200 ul of HCHO.
7. Vortex.
8. Cap tubes and incubate in oil bath @ 100°C for 1 hour.
9. Remove and cool tubes thoroughly to room temperature.
(10) make volumes up to 2.0 ml with 10% TCA.

(11) read samples at A371/443E (or thereabouts) on fluorimeter.

Procedure for Standards:

(1) place 1.8 ml of TCA/FeCl₃ into tubes.

(2) add appropriate amounts of standard (working) solution. (e.g., 10 ul, 25 ul, 50 ul, 100 ul, and blank.)

(3) add 200 ul of HCHO.

(4) Vortex.

(5) cap and incubate in oil bath @ 100°C for 1 hour.

(6) remove and cool tubes.

(7) make volumes up to 2.0 ml with 10% TCA.

(8) read fluorescence @ A371/443E.

For recoveries, known amounts of standard can be added to 25 ul of plasma in 1.8 ml of TCA/FeCl₃. The tubes are then vortexed, spun, and the assay procedure followed as usual. The fluorescence in these tubes is then compared to the fluorescence in tubes containing standard only and sample only (prepared as above).

Remarks: Plastic tubes can be used instead of siliconized tubes provided that they are washed in agua regia every time.
A MODIFICATION OF THE p-DIMETHYLAMINOBENZALDEHYDE REACTION

Introduction

In an attempt to monitor the decrease of tryptophan availability by chemical methods, we encountered difficulties in obtaining reproducibility of the color intensity and maximum of absorption of the p-dimethylamino-benzaldehyde (p-DMA) reaction. Obviously, there was interference in the formation of the blue chromophore complex by the presence of browning pigment (probably the polyketo compounds). As a result of this the color often shifted towards a golden or red hue.

Since not much is known about the chemistry of concentrated sulfuric acid-mediated color reactions and I was also interested in shortening the reaction time of the classical reaction, I developed a modification which is about five times more sensitive than the classical one, and could have application in preliminary protein screening.

Because the activation of tryptophan requires the use of strong HCl, the procedure has two limitations: the color procedure has relatively short stability and corrosive fumes may be harmful to the optics of instruments.

Procedure

The procedure I later discovered, has some similarities with an earlier, less developed procedure (Sullivan, Milone and Everett, 1938).
0.05 ml sample
0.5 ml 20% redistilled HCl
        shake or vortex promptly
0.5 ml p-DMAB soln.
        shake

Red color develops if positive reaction is expected

1.0 ml fresh p-dioxane
        shake

Color development can be accelerated by placing tube in
boiling water bath for up to 5 min.

Read color at 585 NM within 10 min.

Making the p-DMAB Solution

To 15 ml H2O add 50 ml of Baker A.R. H2SO4. Cool
in water bath. Dissolve 1.5 g of p-DMAB while the acid is
still warm. Store protected from light.

Example

Analysis of "Carnation Instant Breakfast." The
food was extracted once with ether and once with CHCl3.
Later it was found that this step was not necessary. One
gram of food was suspended in 10 ml of H2O and the reaction
was carried out as described above.

| Time (min.) | Replicates |
|------------|------------|
|            |            |
| 0          | .440       |
| 5          | .435       |
| 10         | .425       |
| 20         | .445       |
| 30         | .415       |
| 60         | .431       |

O.D. 600 N M
Neither tyrosine nor any other of the 18 common amino acids interfered with the reaction. Phenol, however, was found to give a strong red color of absorption maximum at 550 nm. It is therefore suggested that further study may find applicability of this colorimetric reaction in the determination of phenolic compounds.
APPENDIX C

SUMMARY OF CONCLUSIONS

The problem under study was the loss of the biological properties of a protein when stored with reducing sugars at temperatures below 40°C. The process was referred to as the 'Maillard reaction at mild temperatures,' when there appears to be little or no development of brown color. Emphasis was placed on the temperature factor because marked differences have emerged between the type of damage caused to a Maillard protein depending on the temperature and the stage of the reaction. The conclusions drawn from the foregoing observations were as follows:

1. When insulin was allowed to undergo the Maillard reaction at mild temperature for fifteen days, little or no denaturation took place as evidenced by the high biological activity of the deoxyfructosyl-substituted hormone. Although this conclusion cannot automatically be extended to egg albumin or other food proteins, other pieces of research cited below appear to support the generalization.

2. The small number of sugar residues bound to insulin indicated that, even after four months of storage with glucose, the peptide nitrogen had no participation in the reaction. If that was the case, we would have a situation in which (a) up to forty-nine glucose residues could react with the peptide linkage alone, and (b) reaction of the amide
nitrogen would imply perturbation of a resonant structure which could, in turn, result in the disruption of the tertiary, secondary and primary structures of a polypeptide. Although the presence of free radicals in the Maillard reaction at higher temperatures (Nakimi et al., 1973) could conceivably perturbate the peptide bond or induce polymerization, this did not seem to be the case in the mild browning of insulin. On the assumption that polymeric melanoindins are stable at pH 2.4-2.6 (slightly higher than the gastric pH), one could add with good certainty that no polymeric, reductone-like compounds had yet developed bound to the protein. Clark and Tannenbaum (1974) have lately reported the isolation of limit peptide pigments (LPP) containing at least 8 sugar residues per amino group. Clark and Tannenbaum's LLP's were obtained from an insulin-14C-glucose system reacted at 550 for 37 days.

3. Preferential reaction of the hydrophobic Phe B1 with glucose was consistent with the tertiary and quaternary structures of insulin. Although all amino and carboxylic groups (except for Glu B 13) are available for titration in the hexamer, Phe B1 is generally most reactive towards electrophyllic reagents. The ten-fold increase in acid solubility of the 15-day Maillard insulin does not indicate that the reaction improves the solubility of proteins. Insulin crystals consist of hexamers and packs of hexamers which prevail in aqueous solutions except at very low and high pH.
The hexamers are insoluble because of the predominance of non-polar groups on the surface of the cluster, but the introduction of bulky sugar substituents dissociates the hexamer into soluble dimers.

The second highest reactivity of Gly Al is perhaps due to its less accessible location inside the monomer, whereas the lowest reactivity of B 29 Lys could be explained by high pK of the ε-amino group. The pK value of the guanidinum group of Ang B 22 is so high that it could not be decided if the fourth hexose residue is bound by the guanido or one of the α-amino groups.

4. Since the mild Maillard reaction of proteins does not seem to result in cross linking or oxidative denaturation of the polypeptide chains, the loss of lysine or other essential amino acid by simple undigestibility can be regarded as negligible. Evidence for this comes from (a) the low recovery (6.7%) of 14C-lysine radioactivity in the feces of rats fed 30-day Maillard ovalbumin; (b) the insignificant level of peptide accumulation in the feces of rats fed the 30-day Maillard egg albumin, and (c) the undetectable loss of digestibility for egg albumin even after twenty days of reaction (Tanaka, et al., 1974).

A clear distinction, therefore, must be made between the Maillard reaction catalyzed by high temperatures (55°-121°; processing and autoclaving) and that catalyzed by mild temperatures (ambient - 37°; storage). It is not known
if the results eventually are the same or not, but in the latter case it is possible to observe the initial stages of the reaction. For the case of high-temperature browning it has been reported that up to 70% of the "lysine" radioactivity fed to rats is recoverable in the feces (Valle-Riestra and Barnes, 1970). Also, Clark and Tannenbaum (1974) have reported that pronase digestion of 55°C-Maillard insulin yields undigestible peptides in which B 29 Lys, B1 Phe, A1 Gly, and other residues are involved in the amino-sugar linkages. On the other hand, we have observed that only 67% of the "lysine" radioactivity is recovered in the feces of rats fed mildly brown ovalbumin, and only a few hexose residues were known to react with insulin.

While the role of the intestinal microflora was nil in the degradation of the heavily cross-linked autoclaved Maillard protein of Valle-Riestra and Barnes, it was not clear how significant this effect was in the case of the mildly brown ovalbumin. Considering the almost normal digestibility of Tanaka's Maillard albumin, the known ability of the -N-fructosyllysine products to diffuse across the intestine (without being utilized), and thirdly the ability of our Maillard lysyltryptophan melanoidins to release tryptophan in the intestine, it is possible that little or no action of the microflora was necessary. It should be recalled that, before the one-meal experiment with the $^{14}$C-ovalbumin, there was no induction period with "cold" brown
egg albumin. Table 1 may give an indication that pretreatment of the test rats with brown egg albumin tends to lower even further the recovery of radioactivity. In any event, the mildly brown protein is more highly digested either by the endogenous enzymes or by the microflora enzymes whereas the autoclaved Maillard Protein does not improve digestibility even after suppression of the microflora with antibiotics (Valle-Riestra and Barnes, 1970; Table 2).

5. The sugar-amino acid or protein bond resulting at the initial (Amadori compound) stages of the reaction is quite stable in the presence of both proteolytic enzymes and low pH's (2.4-2.6). This was suggested by the absorption experiments with Maillard glycylleucine and the column chromatography system used for Maillard insulin.
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