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Effect of Maillard Reaction Products on Protein Digestion. Studies on Pure Compounds

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The effects of pure, synthetic compounds, similar to or identical with those formed in the Maillard reaction, on the activity of carboxypeptidase A (EC 3.4.12.2) and purified, intestinal brush border enzyme aminopeptidase N (EC 3.4.11.2) were studied. A number of furans and pyrroles inhibited carboxypeptidase A. The strongest inhibitors had a carboxylic acid as a substituent on the furan ring, and their effects were characterized as an induced substrate inhibition. A few competitive inhibitors of aminopeptidase N were found, the most effective being DL-2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine. This compound also inhibited carboxypeptidase A. When fed to rats (3 mg in a 1-g portion), it reduced the plasma level of lysine coming from the diet.

There are a number of possible physiological implications of the Maillard reaction in food. The reaction may consume the essential amino acid lysine and lower the protein quality. This effect has been extensively studied and is probably the most important of the mechanisms impairing the quality of food proteins during processing (Mauron, 1981; Adrian, 1982). Besides the loss of protein quality, the Maillard reaction may produce compounds that possess other effects on biological systems. These include the production of antibacterial compounds (Einarsson et al., 1983; Anderson et al., 1984; Mester de Parajd et al., 1986), enzyme inhibitors (Öste et al., 1986; Gomyo and Miura, 1986), mutagenic compounds (Shibamoto, 1983; Jägerstad et al., 1986), and nephrotoxic compounds (von Wangenheim et al., 1984; Finot and Furniss, 1986).

In addition to the loss of the lysine, severe heating may reduce protein utilization further, obviously by mechanisms other than the loss of biologically available lysine (Valle-Riestra and Barnes, 1970; Ford and Shorrock, 1971; Pronczuk et al., 1973; Percival and Schneeman, 1979). In two preceding publications we examined the effect of a glucose–lysine reaction mixture per se on the utilization of dietary protein by rats and on the activities of gastrointestinal proteolytic enzymes (Öste and Sjödin, 1984; Öste et al., 1986). We observed that low molecular weight reaction compounds may affect the utilization of dietary protein, possibly due to enzyme inhibition.

In the present paper we report studies on the effect of some pure compounds on the activities on two enzymes, aminopeptidase N (EC 3.4.11.2) and carboxypeptidase A (EC 3.4.12.2). These enzymes were inhibited in vitro by the low molecular weight glucose–lysine reaction mixture (Öste et al., 1986). Aminopeptidase N is located in the membrane of the epithelial cells of the small intestine in swine, rats, and humans (Sjöström et al., 1978; Kenny and Maroux, 1982; Tobey et al., 1985), constitutes a considerable portion of the membrane protein (Sjöström et al., 1978), and may play a central role in the final digestion of peptides escaping the action of pancreatic enzymes, as observed with rat small intestine specimens (Friedrich et al., 1980a,b).

The experiments were performed essentially with various substituted pyrrols and furans that are structurally related to compounds formed in the Maillard reaction. A few inhibitors of aminopeptidase N were found as well as a number of inhibitors of carboxypeptidase A. We also observed that a competitive inhibitor of aminopeptidase N affected the dietary protein utilization, when fed to rats.

EXPERIMENTAL SECTION

Materials. Compounds (structures and names given in Table I) **1** (Miller et al., 1984), **2** (Olsson et al., 1978), **4** (Miller and Olsson, 1981), **5** (Silverstein et al., 1963), **8** (Clemo and Ramage, 1931), **13** (Olsson et al., 1977), **14** (Rapoport and Volcheck, 1956), and **20** (Finot and Mauron, 1969) were prepared according to the literature. Compound **7** was obtained by saponification of the ester ethyl 2,5-dimethylpyrrole-1-acetate prepared as described (Dann and Dimmling, 1953). Compound **12** was prepared from commercial 5-methylfurfural by the Cannizzaro reaction. The other compounds shown in Table I as well as all other chemicals used were commercial samples of analytical grade.

Each compound was dissolved in a small quantity of methanol prior to assaying its enzyme inhibitory effects. This addition of methanol did not affect the enzymes at the conditions of the assays.

Enzyme Assay. Sources of the enzymes and the assay procedures for carboxypeptidase A and aminopeptidase N were as described earlier (Öste et al., 1986). The effects of the compounds on enzyme activities were tested with 0.1–0.8 mM solutions of compound in the assay mixtures, to which the enzymes were added and immediately assayed.

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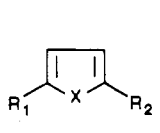
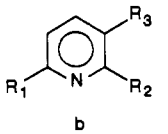
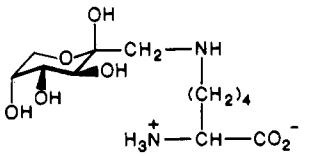
Table I. Structures, Names, and Observed Enzyme Inhibitory Capacities of Various Compounds Formed in, or Similar to Those Formed in, the Maillard Reaction[a]



a



b



20

compd	structure a X	R_1	R_2	name	inhibition (K_i, mM) carboxypeptidase A	aminopeptidase N
1	$N(CH_2)_4CH(NH_2)CO_2H$	CHO	CH_2OH	DL-2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine	**	competitive (0.2)
2	NH	CHO	CH_2OH	5-(hydroxymethyl)pyrrole-2-carboxaldehyde	*	–
3	NH	CHO	H	pyrrole-2-carboxaldehyde	**	–
4	NH	CHO	CHO	pyrrole-2,5-dicarboxaldehyde	–	–
5	NH	CHO	CH_3	5-methylpyrrole-2-carboxaldehyde	*	–
6	NH	$COCH_3$	H	2-acetylpyrrole	**	–
7	NCH_2CO_2H	CH_3	CH_3	2,5-dimethylpyrrole-1-acetic acid	*	competitive (0.6)
8	NCH_2CO_2H	H	H	pyrrole-1-acetic acid	–	competitive (4)
9	O	CHO	CH_2OH	5-(hydroxymethyl)-2-furaldehyde	*	–
10	O	CHO	H	2-furaldehyde	**	–
11	O	CO_2H	H	2-furoic acid	***	weak
12	O	CO_2H	CH_3	5-methyl-2-furoic acid	***	weak
13	O	CO_2H	CH_2OH	5-(hydroxymethyl)-2-furoic acid	***	–

compd	structure b R_1	R_2	R_3	name	inhibition carboxypeptidase A	aminopeptidase N
14	H	CH_3	OH	2-methyl-3-pyridinol	–	–
15	CH_3	H	OH	6-methyl-3-pyridinol	*	–
16	H	CH_3	H	2-methylpyridine	*	NA

compd	name	inhibition carboxypeptidase A	inhibition aminopeptidase N	compd	name	inhibition carboxypeptidase A	inhibition aminopeptidase N
17	levulic acid	*	–	19	α-hydroxypropionic acid	NA	weak
18	propionic acid	–	NA	20	1-deoxy-1-2-*N*-(L-lysino)-D-fructose	–	–

[a] Strength of inhibition of carboxypeptidase A is proportional to the number of asterisks: –, no inhibition; NA, not assayed. Conditions of enzyme assays as reported in Öste et al. (1986).

Animal Experiments. A balanced, purified radioactive diet containing L-[4,5-^{3}H]lysine (80 Ci/mmol) and L-[U-^{14}C]lysine egg white (10 500 dpm/mg) was prepared as described (Öste and Sjödin, 1984), with the exception that all protein was supplied as egg white protein. The radioactivity in the diet was 6950 dpm of ^{3}H/mg and 1090 dpm of ^{14}C/mg. It was used as control diet and for the preparation of the experimental diets. These were obtained by the addition of compound 1 or compound 11 at a concentration of 3 mg/g.

Individually caged, overnight-fasted male Sprague–Dawley rats, 100–110 g, were in the morning fed 1 g of one of the experimental diets or the control diet. After 3 h, the rats were anesthetized and blood was obtained by heart puncture and collected in heparinized test tubes. The radioactivity in plasma was determined by liquid scintillation counting after mixing with Instagel (Packard Instrument Co.).

RESULTS

A number of the tested heterocyclic compounds inhibited the activity of carboxypeptidase A in a way that could not be explained by conventional kinetic theory of enzyme–inhibitor interactions (Dixon and Webb, 1966). The inhibition by a certain concentration of the inhibitor increased strongly when the substrate concentration was raised; i.e., a substrate inhibition was induced by the inhibitor (Figure 1). Attempts to fit the measurements to a straight line in the Lineweaver–Burk plot were unsuccessful. However, as judged by comparison of the shape of the graphs of velocity vs. substrate concentration, the strength of inhibition obtained with the different compounds could be roughly graded as shown in Table I. The gradation could be related to the structure with notable features as follows: Furans that had a carboxylic group as substituent were strongly inhibitory. The twofold symmetrical compounds 4, 7, and 8 had low or no inhibitory effect regardless of the substituent polarity. Com-

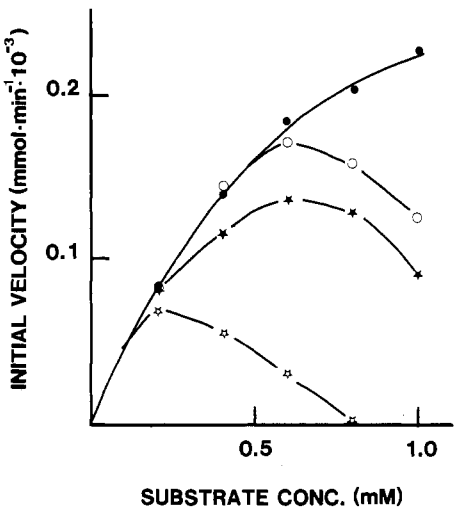


Figure 1. Effect of additions of 2-furaldehyde (0.4 mM, ★), 5-(hydroxymethyl)-2-furoic acid (0.1 mM, ☆), or 6-methyl-3-pyridinol (0.4 mM, ○), on the velocity of hydrolysis of *N*-hippuryl-L-phenylalanine by carboxypeptidase A in 0.1 M sodium borate, pH 7.6, 0.3 M NaCl at 30 °C. ●: no addition.

Table II. Radioactivity in Plasma of Rats 3 h from Feeding an Experimental Diet Containing Radiolabeled Free (^{3}H) and Protein-Bound (^{14}C) Lysine with or without the Addition of Single Maillard Reaction Products

	^{3}H	^{14}C	^{14}C/^{3}H
control diet (7)[a]	29057 ± 1365	12646 ± 940	0.438 ± 0.020
addn of 1 (7)	26307 ± 2186*	11589 ± 819*	0.427 ± 0.037
addn of 11 (7)	27877 ± 1871	11860 ± 720	0.442 ± 0.031

[a] Values are mean dpm/mL ± standard deviation. Asterisks indicate values significantly parentheses: number of rats. Asterisks indicate values significantly different from control ($p < 0.05$, Student's t-test).

pound 1, having a norleucine residue bound to the pyrrole nitrogen, inhibited somewhat stronger than the N-unsubstituted compound 2.

Compounds 1, 7, and 8 inhibited aminopeptidase N significantly. The inhibitions were of competitive type, and the calculated inhibition constants are given in Table I. In addition, a few weaker inhibitors were found (compounds 11, 12, and 19). We also observed that compounds 1 and 7 were the only inhibitors stronger than lysine, which was a competitive inhibitor with a K_i of approximately 2 mM.

The results from the in vivo experiments on rats with compounds 1 and 11 are shown in Table II. A small but statistically significant reduction of the plasma level of both ^{3}H and ^{14}C was observed with 1 in the diet, when compared to the control. No significant effect was observed with compound 11. The ^{14}C/^{3}H ratio was about the same in all three groups. The results suggested a reduced utilization of dietary protein-bound and free lysine, induced by the presence of compound 1 in the diet.

DISCUSSION

The Zn-containing exopeptidase carboxypeptidase A catalyzes the hydrolysis of peptide bonds next to the carboxylic end of peptides and proteins, preferentially when the side chain of the terminal amino acid is hydrophobic as in phenylalanine, tryptophan, or leucine (Neurath et al., 1970). This specificity may be attributed to the presence of a hydrophobic pocket in the active site of the enzyme (Lipscomb et al., 1970). A number of inhibitors of carboxypeptidase A have been reported. These may be aromatic, aliphatic, hydrophobic, or partly hydrophilic compounds with or without an adjacent carboxyl group (Adelman and Lacko, 1968; Davies et al., 1968a; Vallee et al., 1968). The type of inhibition differs with the type (ester or peptide linkage; Auld and Holmqvist, 1974) and the size (Auld and Vallee, 1970) of the substrate molecule. Some compounds possess both inhibitory and activating properties (Davies et al., 1968a).

The previously reported effects of a glucose–lysine reaction mixture on protein utilization in vivo and proteolytic enzyme activities in vitro suggests the presence of low molecular weight enzyme inhibitors in this mixture (Öste and Sjödin, 1984; Öste et al., 1986). During the Maillard reaction numerous hydrophobic, aromatic, and aliphatic compounds are formed from dehydration products of the initially formed, highly hydrophilic reaction products of reducing carbohydrates and amines (Mauron, 1981). It seemed thus not unreasonable to expect that some of these compounds are inhibitors of carboxypeptidase A. For example, the formation of keto acids is consistent with the generally accepted sequences of reactions, and levulinic acid have been isolated in Maillard reaction mixtures (Feather and Harris, 1973; Olsson et al., 1978). A number of keto acids, including levulinic acid, have been shown to inhibit carboxypeptidase A (Adelman and Lacko, 1968). However, as was reported (Öste et al., 1986), carboxypeptidase A was inhibited by a number of fractions of a gel-chromatographed glucose–lysine reaction mixture, the degree of inhibition being roughly proportional to UV absorbance. Thus, the presence of aromatic inhibitors of various molecular weight was indicated, and one compound, DL-2-formyl-5-(hydroxymethyl)pyrrole-1-norleucine, was established as the major one in a strongly inhibiting fraction. In the present study we have shown that this compound inhibited carboxypeptidase A, as did a number of other heterocycles. Among these, compounds **1**, **2**, **5**, **6**, **9**, **11**, **13**, **15**, and **17** are formed in the glucose–lysine reaction (Miller et al., 1984). Compounds **3** and **10** are formed in the Maillard reaction if a pentose is present. Compound **16** has been found in a lactose–casein browning system (Ferreti et al., 1970). The evidence thus shows that aromatic Maillard reaction products may be inhibitory to carboxypeptidase A.

The kinetic parameters of the inhibition could not be calculated. Since the hydrolysis by carboxypeptidase A of N-substituted dipeptides does not follow the classical Michaelis–Menten kinetics (Davies et al., 1968b) and the course of events during catalysis is assumed to be of a multistep nature (Lipscomb, 1980), this may not be too surprising. The substrate inhibition is in fact a feature observed with at least some of the often employed substrates of carboxypeptidase A (Davies et al., 1968b). However, it was outside the scope of the present investigation to precisely describe the nature of the inhibitor–enzyme interactions, and the results will only be considered in relation to the nature of the Maillard reaction.

Furans and pyrroles of the principle structure shown in Table I (structure a) are commonly found products of the Maillard reaction. The furans are generated also at the carmelization reaction of pentoses and hexoses in the absence of amines, although the presence of amines catalyzes their formation (Feeney et al., 1975). The most notable inhibition was observed with 5-(hydroxymethy)-2-furoic acid (compound **13**) which at 0.1 mM concentration abolished the activity of carboxypeptidase A at high substrate concentration (Figure 1). This compound may be formed from 5-(hydroxymethyl)-2-furaldehyde (compound **9**) during conditions favoring oxidation (Turner et al., 1954). The latter compound, found to be a weak inhibitor, is one of the major components formed during nonenzymatic browning in food. The simple pyrroles with unsubstituted nitrogen and the pyridinols (compounds **2**, **3**, **5**, **6**, **14**, and **15**) are generally assumed to be formed via a reaction pathway that includes Strecker degradation of amino acids having a free carboxylic acid group (Nyhammar et al., 1983). Since the concentrations of free amino acids are low in most foodstuffs, the majority of amino groups that may participate in the Maillard reaction is probably those of the protein. Thus the formation of N-unsubstituted pyrroles and pyridinols may be less frequent in real food system. Their presence cannot be completely excluded, however, since during the heating of proteins ammonia is liberated (Bjarnason and Carpenter, 1970) and may form N-unsubstituted heterocycles via direct condensation with carbonyl compounds.

The results showed that the N-substituted pyrrole 1 was a somewhat more effective inhibitor than the unsubstituted analogue **2**. This is of particular interest since this type of pyrrole may be readily formed in comparatively high amounts when the reacting amino compound cannot participate in the Strecker degradation, as has been shown by model studies with glucose and methylamine or glycine (Olson et al., 1977, 1978). Thus, such pyrroles may be

formed and may actually be linked to protein N in foods and eventually be liberated during digestion.

The formation of pyrroles and pyridinols during the Maillard reaction is not only dependent on the original reactants but also on the reaction conditions. As shown in model studies (Nyhammar et al., 1983), the concentrations of pyrroles pass through a maximum during the course of the reaction and are consumed during later stages, while the pyridinols are accumulated. The effect of pH on the yield differs with the type of pyrrole or pyridinol being formed, and some types are evidently favored by a pH close to that of foodstuffs (Nyhammar et al., 1983). However, if not specifically analyzed, it is impossible, at the present state of knowledge, to predict the amount of a pyrrole of furan that is generated in a specific heated foodstuff. Studies of model systems (Olsson et al., 1977, 1978; Miller et al., 1984) indicate that the amount of any specific pyrrole or furan is very low compared to the amount of the reacting carbohydrate.

A few of the compounds were inhibitors of aminopeptidase N. The inhibitions were of a competitive nature. Of the effective compounds, only compounds 1 and 11 have been found in the Maillard reactions. These as well as the weak inhibitors 12 and 19 all have a free carboxylic acid group. Carboxylic groups found in Maillard reaction products may originate from a reacting free amino acid, though its carboxylic group should preferably be lost as carbon dioxide in the Strecker degradation. However, as in the protein the carboxylic acid forms part of the peptide linkage; it is possible that compounds such as DL-formyl-5-(hydroxymethyl)pyrrole-1-norleucine (1) may be formed from protein-bound lysine. Carboxylic groups may otherwise be formed in the Maillard reaction by oxidation of aldehydes or possibly through hydrolytic or oxidative fission of dicarbonyls (Machell and Richards, 1960; Olsson et al., 1978).

To what degree an inhibitor of aminopeptidase N may reduce the protein digestion in vivo was tested in the study with radiolabeled free [^{3}H]- and protein-bound [^{14}C]lysine in the diet. The addition of compound 1 to the diet leads to a reduced level of both labels in plasma 3 h later. This compound inhibited both aminopeptidase N and carboxypeptidase A in vitro. With the administered amount of this compound (3 mg/rat) we assumed that these enzymes should be affected during the in vivo conditions as well. The observed effect cannot, however, be completely explained by an effect on these enzymes during digestion, since the ratio of ^{14}C to ^{3}H did not change significantly; i.e., free lysine in the diet was affected to about the same extent as protein-bound lysine. The result is, however, reasonable if there also was, in addition to an inhibition of the protein digestion, an effect on the intestinal uptake of lysine. Structurally, compound 1 is a neutral amino acid, and it may interfere with the active transport of lysine into the enterocytes, as such an effect has been observed with the common neutral amino acids (Hellier and Holdsworth, 1975).

Further we point out that an effect on the metabolism of lysine after absorption could affect the concentrations of both labels in plasma to the same degree and give the results shown.

We also fed rats the radioactive diet together with compound 11, which was a stronger inhibitor of carboxypeptidase A and a weaker one of aminopeptidase N, compared to compound 1. The mean values of both levels of labels in the plasma of rats fed inhibitor tended to be lower than the control values, but the differences were not statistically significant (Table II). The results indicate that if there is an effect of compound 11, it is a small one.

An earlier study by us (Öste and Sjödin, 1984), performed with the double-isotope techniques also used here, indicates that a low molecular weight glucose–lysine reaction mixture affects the digestion of protein in vivo. We also showed that this mixture inhibits aminopeptidase N and carboxypeptidase A (Öste et al., 1986). The inhibition of aminopeptidase N observed in that study was of the mixed type, and that of carboxypeptidase A was similar to the type of inhibition we observed with pure compounds in this study. The amount of added inhibitor in the present in vivo study was 3 mg to be compared with 22.5 mg of the low molecular weight glucose–lysine reaction mixture in the earlier study. However, according to the K_m values obtained, the capacity to competitively inhibit aminopeptidase N of the intestine in these experiments should have been considerably stronger with compound 1 than with the glucose–lysine reaction mixture. Still the effect on the digestion of dietary proteins seems more pronounced with the reaction mixture than with the pure compound 1. However, the inhibition of aminopeptidase N caused by that mixture also had a strong noncompetitive element. It may be that this noncompetitive inhibition is of importance for the effect in vivo.

In the search for noncompetitive or mixed-type inhibitors of aminopeptidase N, we studied a number of fractions from a glucose–lysine reaction mixture that had been examined with the primary purpose to isolate and identify new reaction compounds (Miller et al., 1984). However, no such pure compound could be identified.

In conclusion, we have found a number of heterocyclic compounds formed in the Maillard reaction that are strong inhibitors of carboxypeptidase A. A few inhibitors of aminopeptidase N were also found, of which two are formed in the Maillard reaction. The in vivo experiments indicated that such compounds may influence the utilization of dietary proteins, if present in sufficient amount in the diet.

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Registry No. 1, 95464-27-0; 2, 67350-50-9; 3, 1003-29-8; 4, 39604-60-9; 5, 1192-79-6; 6, 1072-83-9; 7, 109960-17-0; 8, 19167-98-7; 9, 67-47-0; 10, 98-01-1; 11, 88-14-2; 12, 1917-15-3; 13, 6338-41-6; 14, 1121-25-1; 15, 1121-78-4; 16, 109-06-8; 17, 123-76-2; 18, 79-09-4; 19, 50-21-5; 20, 88125-05-7; carboxypeptidase A, 11075-17-5; aminopeptidase N, 9054-63-1.

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Characterization of Human Colostral Xanthine Oxidase

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Human colostral xanthine oxidase was separated from the milk fat globule membrane and purified by standard techniques. Anion-exchange chromatography yielded XO activity peaks, which presented two active bands on disc-PAGE and ten active bands on isoelectric focusing. Cation-exchange chromatography yielded two XO activity peaks, which presented three active bands on disc-PAGE and nine active bands on isoelectric focusing. Analysis for isoenzymes confirmed the disc-PAGE results that suggested the two isolates contain both molecular size and charge isoenzymes. Subunit analysis found weights between 30 and 250 kDa. The two samples contained primarily acidic variants and the same cofactor ratios, namely, four atoms of iron, four atoms of sulfur, and one atom of molybdenum per molecule of flavin adenine dinucleotide. Treatment with neuraminidase indicated that *N*-acetylneuraminic acid was bound to human colostral XO and was not essential for catalytic activity.

Bovine milk is a rich source for xanthine oxidase (XO; E.C. 1.2.3.2), and a recent study showed that XO is the second most abundant protein on the bovine milk fat globule membrane (Burnier and Low, 1985). While one of the well-known functions of XO is the oxidation of purines, this does not merit its presence at such a high level. In fact, since its discovery over 100 years ago, investigators have attempted to explain the biological role and significance of the enzyme. Recent studies have implicated oxygen-derived free radicals to be the primary mediators of changes in vascular permeability associated with intestinal ischemia. Roy and McCord (1982) observed ischemia-induced conversion of xanthine dehydrogenase (E.C. 1.2.1.37) to XO, while Parks and Granger (1983) suggested the principle source of superoxide anions produced during intestinal ischemia to be XO. The superoxide radical may disproportionate to form hydrogen peroxide, which can, in turn, react with the superoxide in the presence of chelates to form the highly reactive hydroxyl radical (McCord and Day, 1978). The integrity of capillaries is presumed to be altered by this reactive radical via lipid peroxidation. In this process, hydroxyl radical removes an allylic hydrogen from a polyunsaturated fatty

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