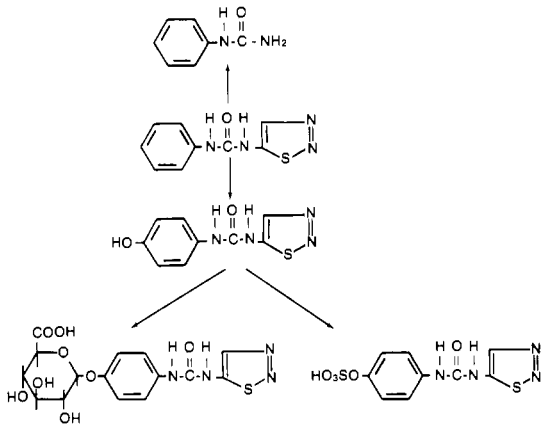

Figure 8. Major paths for thidiazuron metabolism by rats.

as a thidiazuron metabolite from goat urine in our laboratory (Benezet et al., 1978), apparently formed by cleavage of the N–C bond between the thiadiazole ring and the urea bridge. Many substituted urea herbicides, such as diuron and fluometuron, form phenylureas by oxidative N-dealkylation (Geissbühler et al., 1975). Phenylurea obviously cannot be formed from thidiazuron by dealkylation; hence, its formation in goats and rats probably represents a novel cleavage for substituted urea herbicides. We currently are studying the mechanism for its formation. There was little, if any, cleavage by rats of thidiazuron at the urea bridge since aniline, thiadiazole amine, and aminophenols were not detected.

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Measurement of Available Lysine in Processed Beef Muscle by Various Laboratory Procedures

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Three procedures were compared for determining available lysine in beef muscle damaged by heat and glucose or by heat alone (Carpenter, Silcock, and an enzymic method using pronase). The pronase procedure was the most sensitive to lysine damage in beef muscle protein caused by early Maillard, advanced Maillard, and protein–protein type reactions. All amino acids released by pronase from the treated materials except tryptophan were determined. Beef muscle meals processed in the presence of glucose had a greatly reduced release of lysine, histidine, arginine, cystine plus cysteine, glutamic acid, and methionine. Although a fall of over 50% of methionine released by pronase was found, there was no evidence of a release of methionine sulfoxide or methionine sulfone from the meat meals. Beef muscle prepared in the absence of glucose showed a low release of lysine, aspartic acid, glutamic acid, serine, and cystine plus cysteine. This indicated that cross-linkage reactions between lysine and asparagine or glutamine had probably occurred.

The nutritive value of a protein depends not only on its content of essential amino acids but also on the availability of these amino acids for absorption and utilization by the monogastric animal. A loss of availability of amino acids can occur by different mechanisms depending on the conditions of processing of the protein.

Heating proteins in the presence of reducing sugars under mild conditions can cause reactions of the sugar with free NH_2 groups of the protein (early Maillard reactions). Heating protein under severe conditions in the presence of reducing sugars causes more advanced Maillard reactions with destruction of some lysine units. Furthermore, according to Carpenter and Booth (1973), inter and intra peptide linkages can occur during advanced Maillard reactions causing a drop in the availability of amino acids and the general digestibility of the protein. In the absence of sugars, heating of proteins may produce many cross-linking reactions, particularly between lysine and glutamine or lysine and asparagine (Hurrell et al., 1976). According to Carpenter and Booth (1973) such cross-linkages in heated protein very likely hinder digestion in the animal.

Methods using pronase to predict damage of amino acids in processed foodstuffs have been described by Provansal et al. (1975) and Rayner and Fox (1976). Pronase released lysine has been shown to have excellent potential in measuring available lysine in rapeseed meal by its comparison with the Silcock procedure (Rayner and Fox, 1976). However, Hurrell and Carpenter (1974) have shown that the Silcock procedure does not measure lysine availability

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accurately in proteins containing early Maillard products. As the rapeseed meal may have contained early Maillard products, further experiments were required to determine the potential of the pronase procedure. Investigations of the Carpenter available lysine procedure have shown that it measures lysine availability accurately in proteins containing early Maillard, advanced Maillard, and protein cross-linking products (Hurrell and Carpenter, 1974). Therefore to determine whether pronase can predict lysine availability in proteins containing all these reaction products, a comparison was made with values from the Carpenter available lysine procedure, using beef muscle protein as the test material. Beef muscle protein was processed to produce early Maillard, advanced Maillard, and protein cross-linking damage, using similar processing conditions as those described by Hurrell and Carpenter (1974).

The usefulness of the Silcock method for measuring available lysine in processed beef muscle was also determined by comparison with the Carpenter available lysine method.

In addition, the effect of processing damage on the other amino acids was determined by comparing their release by pronase to the release from the untreated beef muscle.

EXPERIMENTAL SECTION

Materials and Methods. One kilogram of lean psoas, major muscle of cattle (beef muscle), was minced and then lyophilized. Fat was removed with diethyl ether in a Soxhlet extractor. The residual protein was ground and passed through a 500-μm mesh screen.

Protein damage of the prepared beef muscle was produced by three different treatments:

(a) Twenty grams of beef muscle was mixed with an equal weight of A.R. glucose and made into a slurry with water, mixed vigorously, and then lyophilized. The material was adjusted to 15% moisture, by slowly adding water during thorough mixing and grinding in a circular blade grinder. The material was placed in a screw-cap jar and placed in a 40 °C incubator oven for 12 days. This processing produces protein damage mainly of the early Maillard type. A large furosine peak was obtained during total lysine determination of this sample, indicating the presence of early Maillard compounds (Hurrell and Carpenter, 1974).

(b) A quantity of 20 g of beef muscle was treated in the same way as described in a. The treated material was placed in a screw-cap jar and denatured in a pressure cooker at 103 kPa and 121 °C for 15 min. Protein damage mainly of the advanced Maillard type is obtained during this processing (Hurrell and Carpenter, 1974).

(c) A quantity of 20 g of beef muscle was placed in a screw-cap jar and denatured in a pressure cooker at 103 kPa and 121 °C for 12 h. Protein damage resulting from interactions between peptides occurred under these conditions.

After the above treatments the samples were lyophilized, ground to pass through a 500-μm mesh screen and stored in a freezer at −20 °C until analyses were carried out.

Amino acids released by pronase and lysine determined by various procedures were analyzed in duplicate on all the treated samples and on an untreated sample of beef muscle.

Total Lysine. Lysine was determined by the method of Spackman et al. (1958) using a Beckman 116 amino acid analyzer. Beef muscle samples of 200 mg were hydrolyzed using 6 N HCl in screw-cap tubes under an atmosphere of nitrogen at 110 °C for 24 h according to the method of Finlayson (1965).

Silcock Available Lysine (Difference Method). Silcock lysine was determined by the method of Roach et al. (1967). Total lysine is determined by acid hydrolysis and a free lysine fraction remaining in solution is subtracted after a separate hydrolysis of protein treated with 1-fluoro-2,4-dinitrobenzene (FDNB). This represents the lysine in the protein which has a free ϵ-NH_2 group and is termed available lysine.

Carpenter Available Lysine (Direct Method). The procedure used was that of Booth (1971) in which the beef muscle was treated with FDNB, hydrolyzed with 6 N HCl and the resulting dinitrophenyllysine (DNP-lysine) measured colorimetrically. This represents the lysine in the protein which has a free ϵ-NH_2 groups. Results from this procedure were calculated using both the correction factors of 1.09 (Hurrell and Carpenter, 1974) and those obtained by determining the loss of added DNP-lysine during acid hydrolysis (Booth, 1971).

Pronase Amino Acids. Amino acids released from beef muscle by pronase were determined as described previously (Rayner and Fox, 1976). Cystine and cysteine were determined in enzyme digests after oxidation with performic acid to cysteic acid (Moore, 1963) and were expressed as grams of cysteine/16 g of N.

Since pronase does not release all amino acids from protein, the pronase procedure remains a relative test. Therefore a reference standard represented by the untreated beef muscle was used in the measurement of availability. The amino acid availability of the untreated beef muscle was assumed to be 100%.

Nitrogen. Nitrogen was determined in all samples by the Kjeldahl method (AOAC, 1960).

Carbohydrates. Glucose and glycogen in the untreated beef muscle were analyzed by the method of Hefferan and Goodnight (1969).

A preliminary experiment was conducted to determine the effect of glucose concentration in the samples upon the release of amino acids by pronase. It was found that the amino acids released were independent of the glucose concentration.

RESULTS AND DISCUSSION

The laboratory prepared beef muscle contained 96% crude protein (N × 6.25% DM), 0.9% crude fat (% DM) less than 0.2% glucose and no glycogen. Because the glucose level was low, the beef muscle was considered suitable for production of processing damage of the protein–protein type.

Total lysine, Silcock available lysine, Carpenter available lysine, and the pronase-released lysine determined in the prepared materials are shown in Table I. The ratio of treated to untreated beef muscle values for Silcock, Carpenter, and pronase lysine tests expressed as a percentage are shown in the parentheses in Table I. The percentage pronase values for treated beef muscle are lower than percentage values obtained from the Carpenter and Silcock methods. This was probably due to the ability of the pronase test to detect changes in overall protein digestibility as well as chemical modifications. Therefore the pronase test may be more sensitive to loss of availability than either the Silcock or Carpenter techniques. This indicates that the pronase test appears to reflect loss of lysine availability due to early Maillard, advanced Maillard, and protein cross-linking reactions.

Although both the Carpenter and Silcock procedures have been reported to correlate closely with animal growth for moderately damaged proteins (Carpenter and Woodham, 1974; Taverner and Rayner, 1975), many experiments have shown poor agreement for severely

Table I. Lysine Values (g/16 g of N) Determined on Treated and Untreated Beef Muscle by Four Different Methods of Analyses

Samples and conditions of treatment	Total lysine	Silcock available lysine	Carpenter available lysine		Pronase lysine
1. Beef muscle	8.68[d]	8.45	8.25[a]	8.41[b]	7.33
2. Beef muscle and glucose stored at 40 °C for 12 days	4.57 (53)[c]	3.18 (38)	2.71 (33)	2.39 (28)	1.29 (18)
3. Beef muscle and glucose heated at 121 °C for 15 min	2.78 (32)	1.21 (14)	1.19 (14)	1.22 (15)	0.68 (9)
4. Beef muscle heated at 121 °C for 12 h	8.09 (93)	6.63 (78)	7.04 (85)	6.85 (81)	5.03 (69)

[a] Values were corrected by recovery factors determined experimentally as 1.07, 1.24, 1.06, and 1.12 for samples 1, 2, 3, and 4, respectively. [b] Recovery factor 1.09 as used by Hurrell and Carpenter (1974). [c] Values in parentheses for the treated samples are percentages of the value for the untreated beef muscle. [d] All values recorded are the mean of duplicate determinations.

Table II. Amino Acids (g/16 g of N) Released by Pronase from Beef Muscle Preparations

Amino acids	Untreated beef muscle	Beef muscle and glucose stored at 40 °C for 12 days	Beef muscle and glucose heated at 121 °C for 15 min	Beef muscle heated at 121 °C for 12 h
Lysine	7.33	1.29 (18)[a]	0.68 (9)	5.03 (69)
Histidine	2.33	1.27 (55)	0.92 (39)	1.83 (79)
Arginine	4.47	2.00 (45)	0.48 (11)	3.73 (83)
Aspartic acid	1.99	1.59 (80)	1.52 (76)	0.84 (42)
Threonine	3.47	2.71 (78)	2.92 (84)	2.72 (78)
Serine	4.99	3.80 (76)	3.82 (77)	3.08 (62)
Glutamic acid	3.61	2.06 (57)	2.00 (55)	2.31 (64)
Glycine	1.48	1.06 (72)	0.96 (65)	1.17 (79)
Alanine	4.45	3.63 (82)	3.56 (80)	3.67 (82)
Valine	4.56	4.00 (88)	4.10 (90)	4.06 (89)
Methionine	3.00	1.45 (48)	1.20 (40)	2.63 (88)
Isoleucine	4.53	3.88 (86)	3.96 (87)	4.13 (91)
Leucine	7.41	6.37 (86)	6.58 (89)	6.65 (90)
Tyrosine	3.25	2.69 (83)	3.06 (94)	2.92 (90)
Phenylalanine	3.55	3.08 (87)	3.14 (88)	3.07 (86)
Cystine plus cysteine	0.47	0.12 (26)	0.01 (2)	0.30 (64)

[a] Values in parentheses for the treated samples are a percentage of the values for the untreated beef muscle. All values are the means of duplicate determinations.

damaged proteins. These studies have indicated that Carpenter available lysine overestimates biological availability in severely damaged protein heated in the presence or absence of sugars (Boctor and Harper, 1968; Amadi and Hewitt, 1975; Carpenter and Booth, 1973; Hurrell et al., 1976). These results support the conclusion that lower values obtained by the pronase procedure in all beef muscle treatments better reflect lysine availability than the Carpenter and Silcock procedures. However, proof of the accuracy of the pronase test for estimating lysine availability is dependent upon agreement with biological availability with these materials.

The experimentally determined correction factors for the Carpenter lysine procedure are shown in Table I. Results for Carpenter available lysine can vary depending on the correction factor used, particularly for the beef muscle heated at 40 °C for 12 days (Table I). As it is not clear which correction factor is valid, either set of Carpenter available lysine values may be correct. Agreement between Carpenter and Silcock available lysine values for the treated beef muscle samples was much better than that obtained by Hurrell and Carpenter (1974) for ovalbumin–lactalbumin mix treated in a similar manner. These results indicate that the Silcock procedure can still be used to measure available lysine accurately in most commercial stockfoods.

The amino acid composition of the untreated and treated beef muscle released by pronase is shown in Table II. Although pronase does not release all amino acids from protein, there was only a small number of insignificant peptides present on the amino acid chromatogram for all beef muscle samples. Also only a trace of cystine was present in enzyme digests for all samples. Beef muscle treated in the presence of glucose showed a loss of all amino acids and particularly a low release of lysine, histidine, arginine, cystine plus cysteine, glutamic acid, and methionine. A number of experiments have shown a loss of all protein-bound amino acids during reactions with sugars (Rao et al., 1963; Sgarbieri et al., 1973), but the mechanism of such reactions, except for the basic amino acids, is not well known. Methionine sulfoxide and methionine sulfone were not evident in the pronase digests from any of the treatments, and consequently methionine in the presence of sugar appears to have been modified by some means other than oxidation. In contrast, Rayner and Fox (1976) have found that considerable quantities of methionine sulfoxide were produced from methionine by autoclaving rapeseed meal. In experiments towards developing an available methionine procedure, Lipton and Bodwell (1976) proposed that the formation of sulfonium derivatives of methionine during heat treatment may be involved in the loss of methionine availability. These products may have been formed in beef muscle processed in the presence of sugars and subsequently caused a fall in methionine released by pronase.

Beef muscle heated in the absence of glucose shows a low release of lysine, aspartic acid, glutamic acid, serine, and cystine plus cysteine (Table II). This indicates that aspartic acid and glutamic acid or more likely asparagine and glutamine (Bjarnason and Carpenter, 1970) may have formed cross-linkages (isopeptide linkages) with lysine which had not been released by pronase. It has been shown that these isopeptide bonds may be as easily digestible as normal peptide bonds (Hurrell et al., 1976).

This means that the pronase procedure appears to exaggerate damage of lysine in beef muscle processed in the absence of sugar, although other forms of lysine damage may have occurred. Only 7% of the total lysine from the beef muscle heated for 12 h in the absence of glucose was not recovered after acid hydrolysis (Table I). This indicates that the fall in lysine released by pronase is mainly due to the formation of inter and intra peptide bonds and not reactions between ϵ amino groups of lysine and breakdown products of other amino acids such as cystine. Serine, which was also released at a low level by pronase from this treatment, may form an ester linkage with aspartic acid and glutamic acid or may have been destroyed. These bonds may be resistant to enzymic attack (Mauron, 1972) and this may explain the low release of aspartic and glutamic acid by pronase.

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An Improved Method for the Quantitation of Limonin in Citrus Juice by High-Pressure Liquid Chromatography

James F. Fisher

Microgram quantities of limonin were resolved from a chloroform extract of citrus juice by high-pressure liquid chromatography using a micro CN column and eluting with a water–methyl alcohol system. The limonin was detected at 210 nm.

Various methods have been published for the determination of limonin (Fisher, 1975, and references therein). The high-pressure liquid chromatographic procedure for the quantitation of limonin in grapefruit juice employing a refractometer reported by Fisher (1975) has the inherent difficulties associated with refractive index detection.

In the method described below, detection is accomplished with an ultraviolet spectrophotometer. The method is more sensitive and factors such as temperature, flow rate, and pressure changes are less critical.

MATERIALS AND METHODS

Apparatus. A Model ALC 202 high-pressure liquid chromatograph (HPLC) with a Model 6000 A pump and U6K injector (Waters Associates, Milford, Mass.) was used. The recorder was a Soltec Model B-281 (Soltec Corp., Encino, Calif.). A Schoeffel UV-visible liquid chromatography analyzer Model SF 770 (Schoeffel Instrument Corp., Westwood, N.J.) was the detector. A Spectra-Physics integrator (minigrator, Spectra-Physics, Santa Clara, Calif.) was used. An International Clinical Centrifuge (Model CL), a Buchi Rotovapor R evaporator, and an ultrasonic cleaner (Cole-Parmer, Model 8845-4) were used.

Column. A Waters Associates 30 cm × 4 mm i.d. μ Bondapak CN column (cyanopropylsilane chemically bonded to a 10 μm porous silica support) was used.

Reagents. The eluting systems were methyl alcohol–water 35:65 or 40:60, v/v. The water was distilled and deionized. The methyl alcohol was Mallinckrodt Spectr AR 3018. Also, a 70:30 isopropyl alcohol–hexane or heptane (Burdick and Jackson) system was used. The solvent systems were degassed with the ultrasonic cleaner.

Sample Preparation. Processed single-strength orange or grapefruit juice or reconstituted concentrate was centrifuged for 5 min at a RCF of 2050 (top speed). A 10-g sample of the supernatant was extracted with 3 × 10 mL of chloroform. The combined chloroform layers were evaporated to dryness in a 50-mL round-bottomed flask, using the rotovapor. The residue was redissolved in 2000 μL of methyl alcohol with the aid of the ultrasonic cleaner.

High-Pressure Liquid Chromatographic Resolution and Quantitation of Limonin. A 20-μL aliquot of the above methyl alcohol solution was injected onto the col-

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