Interaction of Allyl Isothiocyanate with Mustard 12S Protein

N. V. Kishore Kumar Murthy and M. S. Narasinga Rao*

The interaction of allyl isothiocyanate (AIT) with the mustard 12S protein was studied as a function of pH, temperature, ratio of AIT to protein, and duration of interaction, etc. The interaction was found to increase with pH, temperature, and duration of interaction, in the range studied, and it was complete at an AIT to protein ratio of 100 to 1. The electrophoretic mobility of the protein increased with interaction. There was an alteration in the UV absorption spectrum and quenching of the fluorescence intensity. AIT interacted with ϵ-amino groups of lysine and phenolic groups of tyrosine residues. Interaction of the protein with AIT did not affect its hydrolysis by α-chymotrypsin or papain. But, hydrolysis by trypsin is decreased.

INTRODUCTION

Seeds of *Brassica sp.* contain glucosinolates that upon hydrolysis by the enzyme myrosinase yield isothiocyanates that are goitrogenic in nature (Joseffson, 1972). *Brassica juncea* seeds contain predominantely sinigrin (allyl glucosinolate), which yields allyl isothiocyanate (AIT).

Mustard seeds contain both glucosinolate (sinigrin) and myrosinase present in different compartments. Upon flaking and defatting of the seeds, the cell structure is broken and the enzyme comes in contact with the substrate, hydrolyzing it and releasing AIT which can interact with the proteins. Studies with bovine serum albumin (BSA) (Kishore Kumar Murthy, 1982) indicated that the interaction with AIT at neutral pH was negligible. Since the pH of the seed material is about 6.0, the possibility of interaction of the mustard proteins with nascent AIT during oil extraction and isolation of the protein is limited. It is therefore assumed that the proteins isolated from the seed material represent unreacted proteins.

Mustard seed contains two classes of proteins: a high molecular weight fraction (12S) and a low molecular weight fraction (1.3S). The high molecular weight fraction constitutes about 25% of the total proteins, and the low molecular weight fraction constitutes the rest (Gururaj Rao et al., 1978).

Preliminary experiments indicated that AIT interacted with both the 12S and the 1.3S fractions, the interaction with the latter being more pronounced. However, this fraction is heterogeneous and consists of a number of proteins (Lonnerdal and Janson, 1972), whereas the 12S protein appears to consist of only one type of protein that can be fractionated to homogeneity (Gururaj Rao et al., 1978).

In this paper, a study of the interaction of allyl isothiocyanate with the mustard 12S protein is presented.

MATERIALS AND METHODS

Mustard seed (*B. juncea*, variety Varuna RT-59) grown in the year 1978–1979 was obtained from Haryana Agricultural University, Hissar, India.

The dehulled seeds were defatted by repeated extraction with hexane. The defatted meal was ground to pass through an 85-mesh (BSS) sieve and used.

Chemicals from the following sources were used: acrylamide, amido black, ammonium persulfate, and alkali-soluble casein from E. Merck; trypsin, α-chymotrypsin, 2,4,6-trinitobenzene sulfonic acid (TNBS) and [tris(hydroxymethyl)amino]methane (Tris) from Sigma Chemical Co.; allyl isothiocyanate (AIT) and *N,N,N',N'*-tetramethylethylenediamine (TEMED) from Fluka, AG; bis(acrylamide) from Koch Light Laboratories; crystalline papain from the CSIR Centre for Biochemicals. All other chemicals were of analytical reagent grade purchased from BDH or Sarabhai M. Chemicals.

Isolation of the Mustard 12S Protein. The protein was isolated according to the method of Gururaj Rao et al. (1978). The precipitated protein was dissolved in 0.1 M NaCl and dialyzed against the desired buffer before use. The isolated protein was found to be homogeneous by ultracentrifugation and polyacrylamide gel electrophoresis.

Protein Concentration. This was determined spectrophotometrically with $E^{1\%}_{1\,cm}$ 9.9 at 280 nm (Gururaj Rao and Narasinga Rao, 1981).

Allyl Isothiocyanate Concentration. The concentration of a stock solution of AIT in ethanol was determined by measuring the absorbance at 240 nm and using a molar extinction coefficient of 770 (Schwimmer, 1961).

Interaction with AIT. Mustard 12S protein in 0.1 M NaCl was used for all the measurements. The pH of the solution was adjusted to the desired value (pH range 6.0–10.0) by the addition of 1 N NaOH. To this solution were added known amounts of AIT in ethanol, and the pH was maintained constant by the addition of alkali. The reaction was allowed to proceed for 2 h except in the study of the effect of reaction time where the reaction was stopped after different intervals of time. At the end of the interaction period, the pH was decreased to 6.0 by the addition of 1 N HCl and the solution dialyzed against the desired buffer or salt solution.

"Available" Lysine Estimation. This was estimated by the method of Hall et al. (1973), using trinitrobenzenesulfonic acid (TNBS). Lysine was used as a standard.

Tryptophan Estimation. Tryptophan was estimated by the method of Spande and Witkop (1967) using *N*-bromosuccinimide (NBS).

Trypsin Inhibitor Activity. This was determined by the procedure of Smith et al. (1980) using a synthetic substrate, $N\alpha$-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA). A trypsin standard in the absence of the protein was also run.

Spectrophotometric Titration. This was carried out by the procedure of Donovan (1973) using glycine–NaOH buffers in the pH range 8.0–12.6. The number of groups ionized was calculated by using ϵ 2300 for ionized tyrosine (Tanford, 1962).

Fluorescence Spectra. Fluorescence emission spectra were recorded in the range 300–400 nm after excitation at 280 nm, using a Perkin-Elmer fluorescence spectrophotometer, Model 203. A protein concentration of 0.1 ab-

Protein Technology Discipline, Central Food Technological Research Institute, Mysore-570013, India.

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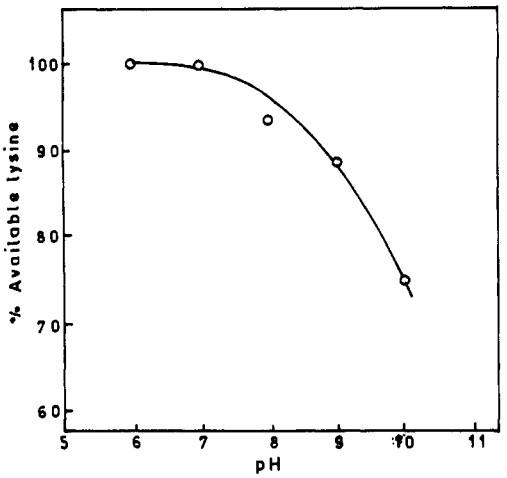

Figure 1. Effect of reaction pH on the available lysine content of mustard 12S protein (ratio of AIT to protein 100; duration of interaction 2 h; temperature 30 °C).

sorbance unit at 280 nm was used.

UV Absorption Spectra. The absorption spectra of the proteins in 0.1 M phosphate buffer of pH 7.6 were recorded in a Perkin-Elmer double-beam recording spectrophotometer, Model 124, in the wavelength range 240–330 nm.

Polyacrylamide Gel Electrophoresis (PAGE). PAGE was carried out in 0.01 M Tris–glycine buffer, pH 8.3, using 6% gels. Electrophoresis was performed for 90 min at a constant current of 4 mA/tube. The amount of protein loaded was 50 μg in each case. The gels were stained for 30 min in 0.5% amido black in 7% acetic acid and destained using 7% acetic acid.

Rate of Proteolysis. The rate of hydrolysis by trypsin, α-chymotrypsin, and papain was measured by the procedure of Gururaj Rao and Narasinga Rao (1981).

All pH measurements were made with a Radiometer pH meter (Titrator TTT-2).

RESULTS AND DISCUSSION

A study of the interaction of AIT with BSA, used as a model protein, indicated that the major reaction involved was a covalent bond formed between the ε-amino groups of lysine and AIT (Kishore Kumar Murthy, 1982). The estimation of "available" lysine content proved a convenient technique to monitor the interaction. Hence, the same technique was used with the mustard protein.

The effect of pH, temperature, AIT concentration, and reaction time on the mustard 12S protein–AIT interaction was investigated.

Effect of pH. This was studied in the pH range 6.0–10.0 with a ratio of AIT to protein of 100 (mole/mole). Studies at pH values higher than pH 10.0 were not carried out since the oligomeric protein may dissociate and also undergo conformational changes at high pH values. Such changes have been reported in the case of other oligomeric proteins (Ishino and Okamoto, 1975; Prakash and Nandi, 1977; Ishino and Kudo, 1979).

The available lysine content did not change between pH 6.0 and 7.0; it decreased at higher pH values (Figure 1). The pH dependence of the reaction of AIT with lysine can be explained on the basis that isothiocyanates are more reactive with the uncharged $-NH_2$ groups than with the positively charged $-NH_3^+$ groups (Edman, 1970; Means and Feeney, 1971).

Effect of Reaction Time. At pH 10.0 and AIT ratio of 100, the reaction was allowed to proceed for different intervals of time, from 5 to 120 min. At the end of each interval, the reaction was arrested by decreasing to pH 6.0.

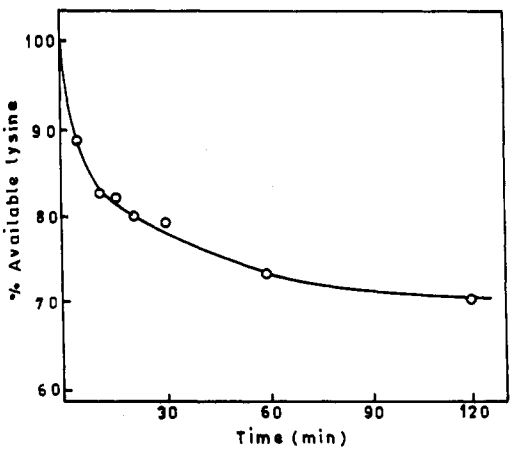

Figure 2. Effect of reaction time on the available lysine content of mustard 12S protein (pH 10.0; ratio of AIT to protein 100; temperature 30 °C).

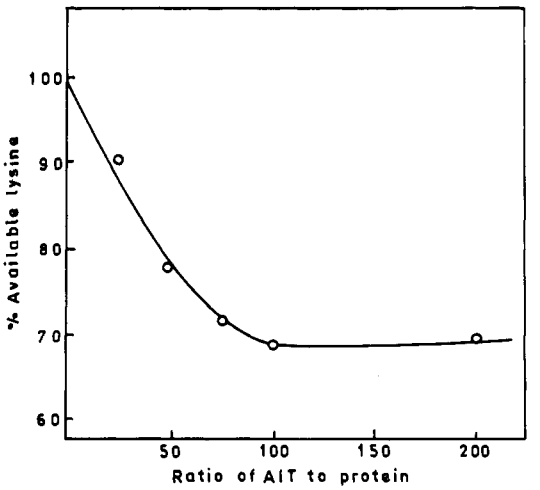

Figure 3. Effect of AIT concentration on the available lysine content of the mustard 12S protein (pH 10.0; duration of interaction 2 h; temperature 30 °C).

The available lysine decreased with an increase in reaction time (Figure 2). After an initial steep reduction in available lysine up to 30 min, a constant value was reached around 120 min, indicating a near completion of the reaction at the end of this duration. Therefore a reaction time of 120 min was chosen for subsequent measurements.

Effect of AIT Concentration. The protein concentration was kept constant, and the ratio of AIT was varied from 25 to 200. The reaction was allowed to proceed for 2 h at pH 10.0.

The available lysine content decreased up to a ratio of 100 and remained constant after this (Figure 3). This suggested that the covalent modifications of most of the available lysines are complete at ratios of ≥100. Hence, a ratio of 100 was used for further measurements.

The above-mentioned measurements were made at 30 °C.

Effect of Temperature. The effect of temperature on the interaction was studied in the range 20–50 °C. Temperatures above 50 °C were not used since it is reported that aggregation of mustard 12S protein occurs above this temperature (Gururaj Rao, 1980). AIT to protein ratio of 100 and a reaction time of 2 h were used.

In this range of temperature the available lysine content decreased with increasing temperature (Figure 4). The increased reactivity may be due to the increase in the kinetic energy of the molecules with temperature, which

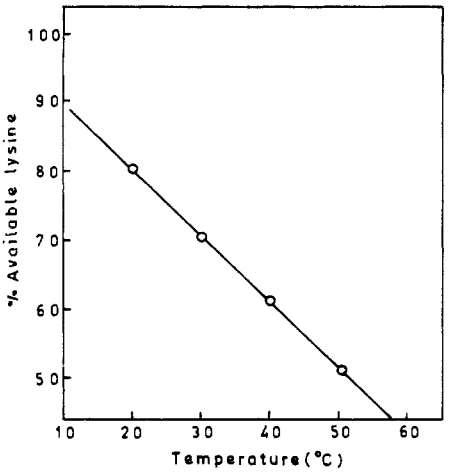

Figure 4. Effect of reaction temperature on the available lysine content of mustard 12S protein (pH 10.0; ratio of AIT to protein 100; duration of interaction 2 h).

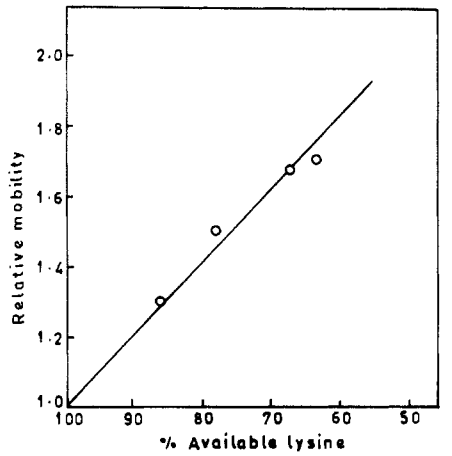

Figure 5. Effect of AIT interaction on the electrophoretic mobility of mustard 12S protein (0.01M Tris–glycine buffer, pH 8.3).

would increase the collision frequency between AIT and the protein molecules. The activation energy, calculated from Arrhenius plot, had a low value of 6 Kcal/mol.

Polyacrylamide Gel Electrophoresis (PAGE). PAGE of native and AIT-interacted mustard 12S protein was carried out in Tris–glycine buffer of pH 8.3. The relative mobility increased with the reduction in available lysine (Figure 5), an observation similar to that made with BSA (Kishore Kumar Murthy, 1982). The increase in mobility is due to increased negative charge on the protein molecule; this is caused by a reduction in the number of ϵ-amino groups of lysine that contribute positive charge. No low molecular weight species were observed, indicating that the interaction had not caused dissociation of the oligomeric protein.

Spectrophotometric Titration. Spectrophotometric titration was carried out with native and AIT-interacted mustard 12S protein where the available lysine was only 70% of the original. The number of phenolic groups titrated was 38 with the native 12S protein and 36 in the AIT-interacted protein (Figure 6).

A reduction of 2 in the number of tyrosyl groups dissociated may not be quantitatively significant if the spectrophotometric titration data are taken alone. However CD measurements also suggested the involvement of tyrosyl groups in the interaction (Kishore Kumar Murthy, 1982). The reduction in the number of tyrosyl residues in AIT-interacted mustard 12S protein is possibly due to the interaction of AIT with the phenolic groups of tyrosine

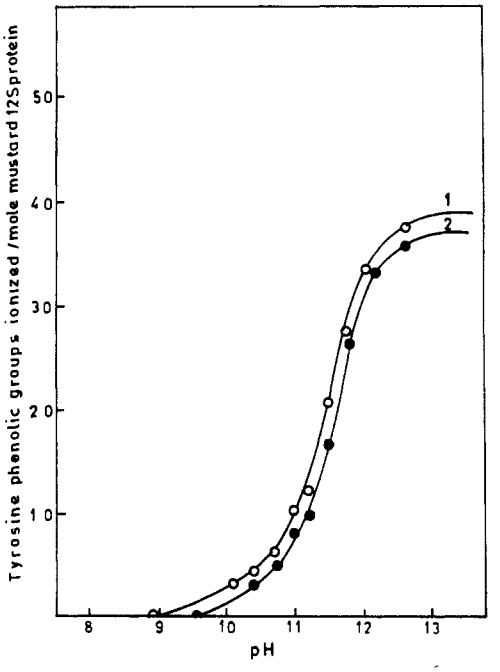

Figure 6. Spectrophotometric titration curve of native and AIT-interacted mustard 12S protein: (1) native mustard 12S protein; (2) AIT-interacted mustard 12S protein (available lysine 70%).

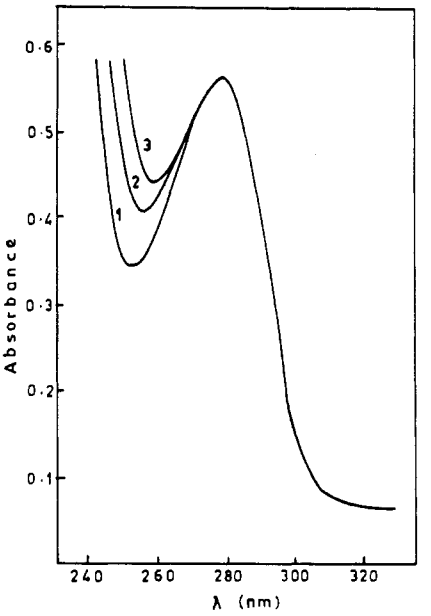

Figure 7. Effect of AIT interaction on the ultraviolet absorption spectrum of mustard 12S protein (0.1 M phosphate buffer, pH 7.6): (1) native mustard 12S protein; AIT-interacted mustard 12S protein; (2) available lysine 86%; (3) available lysine 79%.

residues, as isothiocyanates are known to interact with –OH groups (Drobnica and Gemeiner, 1976). The adduct of AIT with tyrosine would be unstable as in the case of *O*-carbamyltyrosine, which decomposes on dilution or change in pH. It is probable that two tyrosine groups become masked via hydrogen bonding in the AIT-treated protein.

UV Absorption Spectra. The UV absorption spectra of native and AIT-interacted mustard 12S protein with different levels of available lysine content are given in Figure 7. There was no shift in the λ_{max} even though the minimum shifted to higher wavelengths. This may be attributed to the formation of thiourea, which has a very high absorbance around 245 nm (Langer and Gschwend-

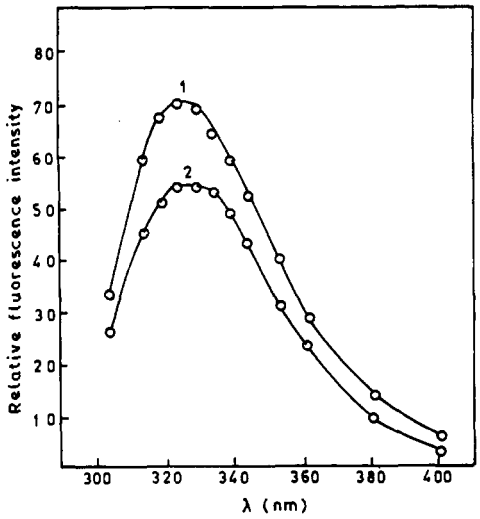

Figure 8. Effect of AIT interaction on the fluorescence emission spectrum of mustard 12S protein (0.1 M phosphate buffer, pH 7.5): (1) native mustard 12S protein; (2) AIT-interacted mustard 12S protein (available lysine 71%).

tova, 1969; Kishore Kumar Murthy, 1982).

Fluorescence Spectra. The native mustard 12S protein gave an emission maximum at 325 nm. Upon interaction with AIT, there was a quenching of the fluorescence intensity (Figure 8).

Tryptophan estimation in the native and AIT-interacted mustard 12S protein (reduction in available lysine 30% and 50%) revealed that there was no change in the number of tryptophan residues upon interaction. Hence, the quenching that was observed was not due to a reduction in the number of tryptophan residues. Possibly interaction with AIT causes a minor change in the conformation of the protein that leads to quenching of the fluorescence emission.

Rate of Proteolysis. The rate of proteolytic cleavage of the peptide bond is sensitive to changes in the conformation of a protein molecule as it depends on the state of localized areas in the molecule (Markus, 1965). This technique cannot, however, delinate any particular conformation but can only detect conformational changes (Anfinsen and Redfield, 1956; Harrington et al., 1959; Mihalyi and Godfrey, 1963). The rate of proteolysis by papain, which is not bond specific, and by two bond-specific proteases (α-chymotrypsin, trypsin) was measured. The measurements were made with the unreacted protein and AIT-treated protein.

Hydrolysis by α-chymotrypsin and papain (Figure 9a,b) showed that the native mustard 12S protein was fairly resistant to hydrolysis. This resistance has been attributed to conformational restraints (Gururaj Rao and Narasinga Rao, 1981). No change in the rate of hydrolysis of the protein was observed upon interaction with AIT.

CD and spectrophotometric titration studies suggested that AIT interacted with tyrosine residues in addition to the ϵ-amino residues. Hydrolysis with α-chymotrypsin, which is specific for bonds formed by carboxyl groups of tyrosine, phenylalanine, and tryptophan, indicated that there was no change in the extent of hydrolysis upon interaction with AIT. This could be due to the fact that only two of the 38 tyrosine residues had been blocked upon AIT interaction and, also, phenylalanine and tryptophan had not interacted with AIT.

Hydrolysis of the protein by papain, which is a nonspecific protease, was also not affected by AIT interaction. Only a few ϵ-amino groups and only two tyrosine residues are modified by AIT interaction, leaving all the other amino acid residues unaffected. This may be the reason for the observation that hydrolysis was not affected.

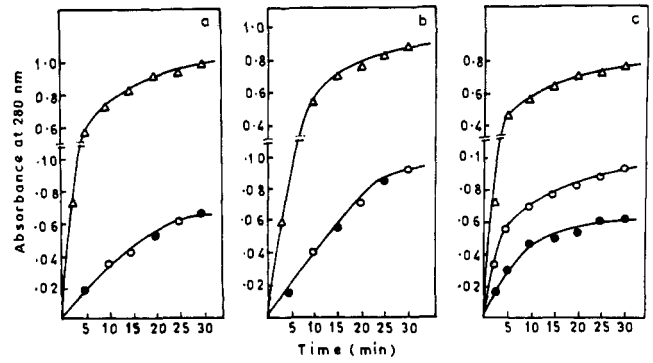

Figure 9. Effect of AIT interaction on the rate of proteolysis of mustard 12S protein: (a) by α-chymotrypsin (0.1 M borate buffer, pH 7.8 containing 0.005 M Ca^{2+}); (b) by papain (0.05 M Tris-HCl buffer, pH 8.0 containing 0.005 M cysteine and 0.002 M EDTA); (c) by trypsin (0.1 M phosphate buffer, pH 7.6). Key: (a) ○, native mustard 12S protein; (b) ●, AIT-interacted mustard 12S protein (available lysine 81%); (c) △, casein.

The mustard 12S protein was poorly hydrolyzed by trypsin also (Figure 9c) and interaction with AIT reduced the hydrolysis even further. Since trypsin is known to be specific for bonds involving arginine and lysine residues, this reduction in the extent of hydrolysis could be attributed to the reduction in the number of ϵ-amino groups after interaction with AIT. Similar results have been reported by a number of earlier workers who used various chemicals to block the ϵ-amino groups of lysine (Anfinsen and Redfield, 1956; Weil and Telka, 1957; Stark and Smyth, 1963).

To determine whether the reduction in the rate of hydrolysis was due to any inhibitory effect of some product of the mustard 12S protein–AIT interaction, measurements were made using a synthetic substrate, BAPNA. Pure AIT or thiourea did not inhibit the hydrolysis of BAPNA by trypsin. However, the mustard 12S protein showed some trypsin inhibitory activity. This inhibition by the protein increased with reduction in available lysine content. Thus, the decreased hydrolysis by trypsin could be a combined effect of the blocking by AIT of the ϵ-amino groups of the lysine residues in the protein and the inhibitory effect of the product of AIT interaction with the mustard 12S protein.

The results of these studies of AIT–mustard 12S protein interaction indicate that AIT interacts covalently with two types of amino acid residues, lysine and tyrosine, and the reactions are pH dependent. No major changes occurred in the structure of the protein, including its quaternary structure. The alteration in UV and fluorescence spectra and the enzymic hydrolysis of the protein is due to the effect of the product(s) of interaction with AIT rather than to any major conformational change.

Registry No. AIT, 57-06-7; L-lysine, 56-87-1; L-tyrosine, 60-18-4; trypsin, 9002-07-7.

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Depletion of Intramuscularly Injected Procaine Penicillin G from Tissues of Swine. A Comparison of HPLC and Bioassay Procedures

William A. Moats,* Elmer W. Harris, and Norman C. Steele

Fifteen cross-bred pigs were treated intramuscularly with 13 200 IU of procaine penicillin G/kg of body weight. The pigs were slaughtered in groups of three 4 h and 1, 2, 4, and 8 days after treatment. Injected muscle, contralateral uninjected muscle, liver, kidney, and blood serum were collected and stored frozen at −20 °C until analyzed. Four hours after treatment highest levels (4.8–525 ppm) were present in injected muscle. Levels in kidney (0.76–2.2 ppm) were higher than in blood serum (0.19–0.32 ppm) while levels in uninjected muscle were variable (0.09–1.9 ppm) and were lower than in blood serum in two of three animals. Twenty-four hours after treatment, blood serum and tissues except injected muscle were free of detectable penicillin G. By 48 h, the injected muscle was essentially free of penicillin. HPLC results were frequently severalfold higher than microbiological assays. Nonspecific microbial inhibitors were frequently found in both tissue and blood serum. Microbial inhibitors inactivated by penicillinase were found in control kidneys from untreated pigs. These inhibitors could be recovered by the cleanup for penicillin G, but penicillin G was not found by HPLC analysis.

The depletion rate of antibiotics from blood and tissues of farm animals following administration is of interest in establishing therapeutic regimens and in assessing potential residue problems. Most studies of depletion of penicillin G in animals have been based on determination of levels in blood serum, and few data are available on distribution in various tissues. The persistence of penicillins administered to animals either intramuscularly (im) or subcutaneously (sc) varied considerably depending on the dosage and the form used. English (1965) observed that increased dosage resulted in increased blood levels to a certain limiting level above which increased dosage resulted in longer maintenance of therapeutic levels in blood. The dosage form was also important. Sodium or potassium salts were rapidly depleted, the procaine salt was more persistent, and benzathine penicillin G was retained markedly longer than the others (Jaksch, 1961; Rolinski and Fidecka, 1962; English, 1965).

Penicillin G was detectable in blood serum of some steers (three of seven) up to 36 h after a single im dose of 33 000 IU/kg of the procaine salt (Mercer et al., 1971a; Teske et al., 1972). Nouws and Ziv (1977) reported detectable levels in blood, muscle, and kidney up to 36 h after im treatment of dairy cows with penicillin G. Levels in renal cortex were higher than in blood serum while levels in muscle drip were lower. Residue ratio was affected by dosage form (procaine vs. sodium salt). Mercer et al. (1971b) found that after im treatment of swine with procaine penicillin G, blood levels peaked 1 h after injection and were barely detectable 24 h posttreatment. Penicillin G concentrations ≥ 0.04 IU/cm^3 were found in blood serum of swine up to 84 h after subcutaneous (sc) injection of 40 000 IU/kg of benzathine penicillin (Jaksch, 1961) and up to 120 h after sc treatment with 20 000 IU/kg of benzathine penicillin G (Rolinski and Fidecka, 1962). Mercer et al. (1978) found that potassium penicillin G infused intravenously (iv) into swine was rapidly cleared from blood and tissues. The longest times penicillin could be detected after treatment were 240 min in blood plasma and kidney, 90 min in lung, spleen, and muscle, and 5 min in liver. Tissue concentrations were well below those in plasma. Bergholz et al. (1980) studied distribution of

U.S. Department of Agriculture, Agricultural Research Service, Animal Science Institute, Meat Science Research Laboratory, BARC-East (W.A.M.), Food Safety and Inspection Service (E.W.H.), and Agricultural Research Service, Animal Science Institute, Nonruminant Animal Nutrition Laboratory (N.C.S.), Beltsville, Maryland 20705.