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

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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

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[^{14}C]benzylpenicillin in rats after ip administration. The tissue/plasma ratios 15 min after treatment were based on ^{14}C distribution: liver, 6.9; kidney, 7.0; muscle, 0.24. Probenicid decreased tissue uptake. Detectable penicillin levels were found up to 120 h in tissues of chickens fed diets with up to 500 ppm of benzathine penicillin (Moreno and Calles, 1980).

Except for distribution studies utilizing radiolabeled compounds, the results obtained in previous studies were obtained by using microbiological assays (MBA). Our laboratory recently developed methods for the quantitation of penicillin G in tissues using high-performance liquid chromatography (HPLC) (Moats, 1984). The present study was undertaken to obtain data on distribution of penicillin G in swine tissues following im treatment with the procaine salt and to compare the HPLC and MBA methods.

METHODS AND MATERIALS

Chemicals and Equipment. *Equipment.* Vortex mixer; Buchler Rotary Evapomix; blender; graduated cylinders, separatory funnels; 15-mL conical centrifuge tubes graduated to 0.1 mL. All glassware was cleaned in special detergents designed for critical cleaning and rinsed with 1% hydrochloric acid and distilled water before use.

Chemicals. Acetonitrile, UV grade (OD(220 nm) $\leq$ 0.02); methylene chloride and petroleum ether (30–60 °C), residue analysis grades; other chemicals, reagent grade.

Procedure. *Preparation of Tissue Homogenates.* About 25 g of tissue was blended for 2 min with 3 volumes (v/w) of distilled water.

Deproteinization. A 5-mL portion of blood serum was mixed with 1 mL of 0.2 M Na_2HPO_4 in a 125-mL conical flask, and 3 volumes (18 mL) of acetonitrile was added with swirling. After the mixture was allowed to stand for 1 min, the supernatant liquid was decanted through a plug of glass wool in the stem of a funnel and a half-volume (12 mL) of filtrate collected. This is equivalent to 2.5 mL of original serum.

Tissue Homogenates. Acetonitrile (32 mL) was added to 8 mL of tissue homogenate as described for blood serum and a half-volume (20 mL) of filtrate collected. This is equivalent to 1 g of original tissue.

Cleanup. The acetonitrile filtrate was transferred to a separatory funnel, and 10 mL of 0.2 M pH 2.2 phosphate buffer was added. The filtrate was then extracted with 20 and 10 mL of methylene chloride, and the combined methylene chloride extracts were collected in a clean separatory funnel. Then, 40 mL of petroleum ether (30–60 °C) and 15 mL of acetonitrile were added to the methylene chloride extract, and the mixture was washed twice with 2-mL portions of distilled water that were discarded. The organic layer was then extracted with four successive 1-mL portions of 0.01 M pH 7 phosphate buffer that were combined in 15-mL graduated conical centrifuge tubes. The pH 7 extract was washed by vortexing 10 s with 2 mL of petroleum ether followed by low-speed centrifugation to separate the layers. The petroleum ether was removed with a pipet as completely as possible, and the remaining petroleum ether was evaporated under reduced pressure on the Evapomix. Then, 1 mL of saturated ammonium sulfate and 2 mL of acetonitrile were added and mixed for 10 s on a vortex mixer. The tube was centrifuged at low speed for 1 min, and the acetonitrile layer was carefully transferred with a pipet to a clean centrifuge tube. The aqueous layer was extracted as above with an additional 2 mL of acetonitrile that was combined with the first extraction. The acetonitrile was evaporated on the Evapomix to a volume of <0.5 mL, which was residual water, and the volume adjusted to 0.5–1.0 mL with distilled water.

HPLC Procedure. The apparatus used was a Varian Model 5000 liquid chromatograph equipped Varian UV-50 variable-wavelength detector set at 220 nm and a Valco automatic loop injector with a 200-μL loop. The column was a Varian Micropak MCH-10 10-μm o.d. reversed-phase column, 4.6 mm × 30 cm. Solvents: (A) 0.01 or 0.02 M phosphoric acid; (B) acetonitrile. A gradient of 80A:20B–40A:60B in 20 min was used with a flow rate of 1 mL/min. Quantitation was based on peak height, which was linear up to 6 μg of penicillin injected onto the column.

Treatment Protocol. Twenty-one cross-bred pigs weighing 80–110 kg with no history of exposure to penicillin or other antibiotics were used. Fifteen of these animals were weighed and injected intramuscularly (ham) with 13 200 IU/kg of procaine penicillin G. The pigs were slaughtered in groups of three at intervals after treatment of 4 h and 1, 2, 4, and 8 days. Pigs were fed and watered ad libitum, prior to and following penicillin treatment. Blood was obtained at slaughter, and injected muscle, contralateral uninjected muscle, liver, and kidney were obtained within 15 min of exsanguination. All tissues were immediately frozen and stored at −20 °C prior to analysis. Blood serum was collected and also stored at −20 °C. Three control pigs were slaughtered at the beginning and at the end of the experiment. Aliquots of the water homogenates of tissues were analyzed in replicate by both HPLC and microbiological assay.

Microbiological Assays. Aliquots of blood serum and tissue homogenates in water as prepared for HPLC analysis were taken for microbiological assay. The test organism used was *Sarcina* (*Micrococcus*) *lutea* (ATCC 9341A). The assay medium was antibiotic medium #2, pH 6.0 (BBL). Duplicate plates were concurrently analyzed with and without 100 000 units of penicillinase (BBL)/mL. The seeded agar was overlayed with Lab-Line bioassay plates (metal plates with wells), 0.2 mL of sample extract was placed in the wells and incubated 16–18 h at 29 °C, and the inhibition zones were measured. Quantitation was based on zone diameter compared with a standard curve of zone diameter vs. concentration of penicillin G. Where inhibitors not inactivated by penicillinase were found, the difference in the apparent penicillin concentrations on the plates with and without penicillinase was calculated and taken as the value for penicillin.

RESULTS AND DISCUSSION

Four hours after treatment, concentrations of penicillin were quite high in the injected muscle (Table I) as would be expected. The levels found in successive samples of the same injected muscle also varied considerably, indicating the penicillin was not uniformly distributed. The concentration of penicillin would be expected to diminish with distance from the injection site in the injected muscle. Levels found by HPLC assay were as much as 100-fold higher than were found by MBA. However, concentrations were probably too high in the injected muscle at this time for accurate determination by MBA without further sample dilution. In the contralateral uninjected muscle (Table II) 4 h after treatment, concentrations found by HPLC were near 0.1 ppm in two of the three animals, which was below blood serum levels, and over 1 ppm in the third, which was well above blood serum levels. Agreement between MBA and HPLC results was better than with the injected muscle. The results also showed uniform distribution in the muscle of animals 234 and 235 but some variability in animal 236. Animal 234 had an inhibitor not inactivated by penicillinase, and this apparently resulted in low bioassay results. Levels of penicillin found in liver

Table I. Penicillin G in Injected Muscle of Swine

animal no. at time posttreat	penicillin G found, ppm					
	sample 1		sample 2		sample 3	
	HPLC[a]	MB[b,c]	HPLC	MB	HPLC	MB
4 h						
234	269	3.3*	46	0.50*	525	4.6
235	17	0.45*	80	0.71*	174	7.2
236	15	0.71*	19	ND	4.8	1.9
24 h						
237	0.22	0.01*	60	12	9.6	3.0
238	1.6	0.18*	3.0	1.3	2.3	1.3
239	0.45	0.03*	0.14	0.06	0.08	0.10
48 h						
240	0.05	0	0	0	0	0
241	0.05	0	0	0	0	0
242	0	0	0	0	0	0
untreated control	0	0*				

[a] HPLC = determination by high-performance liquid chromatography. Mean of replicates. [b] MB = determination by microbiological assay using *Sarcina lutea* with confirmation by penicillinase. Mean of replicates. [c] Key: *, contained natural inhibitor not inactivated by penicillinase; ND, not done.

Table II. Penicillin G in Contralateral Uninjected Muscle from Treated Swine

animal no. at time posttreat	penicillin G found, ppm					
	sample 1		sample 2		sample 3	
	HPLC	MB[a]	HPLC	MB	HPLC	MB
4 h						
234	0.09	0.03*	0.09	0.02*	0.09	ND
235	0.11	0.07	0.11	0.08	0.12	ND
236	1.90	1.30	0.58	1.46	1.03	ND
24 h						
237	0	0.5	0	0		
238	0	0	0	0		
239	0	0	0	0		

[a] *See footnotes, Table I.

Table III. Penicillin G in Liver of Swine Injected Intramuscularly

animal no. at time posttreat	penicillin G found, ppm					
	sample 1		sample 2		sample 3	
	HPLC	MB[a]	HPLC	MB	HPLC	MB
4 h						
234	0.74	0.01*	0.10	0.03*	0.13	0*
235	0.93	0.02	0.19	0.04*	0.18	0*
236	0.46	0	0.14	0.05*	0.04	0*
24 h						
237	0.14	0	0.03	0*	0.03	0.02*
238	0.21	0.05	0.09	0.0*	0.07	0*
239	0.18	0	0.06	0*	0.06	0*
48 h						
240	0	0*				
241	0	0*				
242	0	0*				

[a] *See footnotes, Table I.

(Table III) were higher by HPLC than by MBA. Inhibitors not inactivated by penicillinase were common in liver homogenates. It is possible that penicillin was metabolized by liver homogenates during the incubation period required by MBA.

Kidney tissue (Table IV) showed relatively high concentrations of penicillin G at 4 h. Inhibitors not inactivated by penicillinase were present in most samples, and apparent penicillin was found in the untreated controls. The apparent low levels found after 24 h by MBA were not higher than the controls and are therefore probably not penicillin. The results varied somewhat with successive samples by either assay procedure. While HPLC results

Table IV. Penicillin G in Kidneys of Swine Injected Intramuscularly

animal no. at time posttreat	penicillin G found, ppm					
	sample 1		sample 2		sample 3	
	HPLC	MB[a]	HPLC	MB	HPLC	MB
4 h						
234	2.11	0.70*	2.53	0.84	2.19	0.44*
235	2.18	1.60*	3.33	0.90*	1.52	1.63*
236	1.00	0.58*	0.97	0.47*	0.76	0.77*
24 h						
237	0	0.01*	0	0.03*		
238	0	0*	0	0.07*		
239	0	0*	0	0.02*		
control	0	0.05*				

[a] *See footnotes, Table I.

Table V. Penicillin G in Blood Serum of Swine Injected Intramuscularly

animal no. at time posttreat	penicillin G found, ppm		
	MB[a]	HPLC	
4 h			
234	0.07*	0.19	0.19
235	0.01*	0.32	0.30
236	0.02*	0.27	0.26
24 h			
237	0.02*	0	0
238	0.02*	0	0
239	0.04*	0	0

[a] *See footnotes, Table I.

Table VI. Apparent Penicillin in Frozen Pork Kidney Controls with No Known History of Exposure to Penicillin

sample	treatment	inhibn zone, mm		app penicillin, ppm	HPLC anal. (Pen G)
		init	after penicillinase		
233	pH 8 homog	NI[a]	NI	0	
	homog ctr (supernat)	NI	NI	0	
	acetonitrile extr	10.4	NI	0.025	
	HPLC cleanup	20	NI	0.10	neg
250	pH 8 homog	11.4	NI	0.03	
	homog ctr (supernat)	17.0	NI	0.07	
	acetonitrile extr	12.6	NI	0.04	
	HPLC cleanup	21.2	NI	0.12	neg
249	pH 8 homog	14.4	NI	0.05	
	homog ctr (supernat)	14.0	NI	0.04	
	acetonitrile extr	NI	NI	0	
	HPLC cleanup	18.4	NI	0.08	neg

[a] NI = no inhibition.

were higher on the average than those of MBA, there was no consistent relationship and some MBA results were comparable to or higher than by HPLC. The levels found in kidney were about 4–12-fold higher than the corresponding blood serum levels.

Blood serum (Table V) samples all contained inhibitors not inactivated by penicillinase. MBA results at 4 h were lower than HPLC and were not different from MBA results after 24 h, at which time the serums were negative for penicillin by HPLC.

By 24 h, all tissues except liver and injected muscle were negative. In injected muscle, levels found by HPLC were still generally higher than were found by MBA although differences were less pronounced than after 4 h. Agreement was better at low levels of penicillin. By 48 h, only

Table VII. Effect of Sample Treatment on Recovery of Penicillin G by Microbiological Assay

inj muscle animal no.	homog med	penicillin G found, ppm: microbiological assays[a]: homogenate: direct	homogenate: centrifuged (supernat)	acetonitrile extr[b]	HPLC cleanup	HPLC anal.
234	0.01 M pH 7 phosphate buffer	213	162	ND[c]	162	544
235	0.2 M pH 2.2 phosphate buffer (final pH 3.4)	5.5	5.1	3.9	45	51
236	0.01 M pH 8 phosphate buffer	3.3	0[d]	ND	4.2	6.0

[a] Diluted 1:500 with pH 6.0 buffer prior to test. [b] Tested after removal of acetonitrile under vacuum. [c] ND = not done, sample lost while removing solvent. [d] Overdiluted prior to test.

two of nine samples of injected muscle showed detectable penicillin and this was near the limit of sensitivity by HPLC.

The only other report of actual tissue levels of penicillin G in swine resulting from therapeutic treatment is that of Mercer et al. (1978) who found little accumulation in tissue and rapid clearance from blood after iv infusion of penicillin G.

Our results suggest that individual pigs may vary considerably in retention of penicillin in tissues. Animal 236 showed much higher levels in uninjected muscle and lower levels in kidney relative to blood serum levels than the other two pigs slaughtered at the same time.

Microbial inhibitors not inactivated by penicillinase were frequently found in tissue homogenates and also in blood serum. Levels of penicillin found by MBA were frequently low in such samples. The presence of natural microbial inhibitors in swine tissue has been reported by others (Vilim and Larocque, 1983; Corry et al., 1983; Ziv and Nouws, 1977). When present, they interfere with accurate determination of low levels of penicillin in tissues (Vilim and Larocque, 1983). Smither (1978) attributed inhibition to the product of bacterial growth in tissues assayed. This could not be true in the present studies since all tissues were immediately frozen after collection and held frozen until they could be analyzed. Corry et al. (1983) reported that frozen but not fresh pig kidneys were especially prone to give false-positive reactions. Corry et al. (1983) and Ziv and Nouws (1977) reported that inhibition zones <1–2 mm were ignored in MBA of animal tissue because of the presence of low levels of natural inhibitors.

Some of the control kidneys contained an inhibitory substance inactivated by penicillinase. Inactivation by penicillinase is commonly considered to be a confirmatory test for β-lactam antibiotics. Experiments were run (Table VI) to determine whether this inhibitor could be eliminated by the cleanup procedure used to prepare samples for HPLC analysis. Two of three kidneys tests showed inhibition in homogenates prepared in 0.01 M pH 8 phosphate buffer. A portion of the homogenate was centrifuged and the supernatant liquid tested. Another portion of the homogenate was treated with 4 volumes of acetonitrile as in the usual cleanup procedure. An aliquot was tested after removing the acetonitrile under reduced pressure. The remainder was carried through the rest of the HPLC cleanup, and this was tested by both HPLC and MBA. The results were variable in the supernatant and the acetonitrile extracts, but inhibition was enhanced after cleanup for HPLC. In all cases, inhibition was totally eliminated on the plates containing penicillinase. No penicillin G was found by HPLC.

There is no reason to suppose that the inhibitory substance found is in fact a β-lactam antibiotic since the control pigs had no known history of exposure to β-lactam antibiotics. Exposure would have had to have been recent since the longest reported time for detection of penicillin G residues posttreatment is 120 h (Rolinski and Fidecka, 1962). Cross-contamination was not a factor since the antibiotic is not penicillin G. Accidental introduction from feed is unlikely since the penicillin G is the only β-lactam commonly used in feeds. β-Lactams, other than those with neutral side chains such as penicillin G and cloxacillin, cannot be recovered by the cleanup used, and so the possible presence of most β-lactams can be ruled out. Recovery by the cleanup procedure used indicates that it is an organic acid. It is likely that it is a natural bacterial inhibitor. The possible occurrence of natural inhibitors inactivated by penicillinase is of considerable importance to regulatory agencies, and it would be of interest to positively identify the compound or compounds. Rieve et al. (1974) found that more than 10% of hog kidneys tested in Germany were positive for penicillin when inactivation by penicillinase was used for confirmation. This result would imply an extraordinarily high use of penicillin in German swince since penicillins clear rapidly from swine tissue. The alternative explanation that substances were present that gave a false-positive test for penicillin must be considered.

An effort was also made to improve agreement between HPLC and bioassay in tissues. Results with three samples of injected muscle are shown in Table VII. These were homogenized in buffer as indicated, and aliquots were centrifuged, treated with 4:1 acetonitrile, and carried through the cleanup for HPLC. All samples were diluted 1:500 with 0.01 M pH 6 phosphate buffer before bioassays were run. Levels of penicillin found by HPLC varied from 6.0 to 544 ppm. Agreement was better with samples homogenized in pH 7 and 8 buffers than was found (Table I) with water homogenates although results were still higher by HPLC. Cleanup had little effect. When samples were homogenized in pH 2.2 buffer, HPLC results were about 10× MBA results except after final cleanup where agreement between the two methods was good. Quantitation of bioassays was based on a calibration curve of inhibition zone diameter vs. concentration. This was not linear and was therefore less accurate outside of the middle range of the plot. Accuracy might be improved by using a number of dilutions of samples so that at least one would be within the linear range for quantitation.

Although this study showed that penicillin G was rapidly cleared from swine tissues at the recommended dose levels used, reports in the trade press (Aldrich, 1984) indicate that much higher dosages are frequently used. Reports cited previously indicate that retention time in blood serum is increased at higher dosage levels, by use of the benzathine salt, and also by the use of sc rather than iv or im injection (Jaksch, 1961; Rolinski and Fidecka, 1962; English, 1965). It would be of interest to determine the effects of varying the dose form and level and mode of administration on residues in tissues.

Registry No. Penicillin G, 61-33-6.

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Isolation of Coumarin in Snap Beans and Its Effect on Uredospore Germination

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Coumarin was isolated and identified for the first time from leaves and pods of five cultivars of snap beans (*Phaseolus vulgaris* L.). Isolation of coumarin was by thin-layer chromatography and identification by thin-layer cochromatography, ultraviolet spectroscopy, and mass spectrometry. The maximum concentration of coumarin in all leaf and pod tissues analyzed never exceeded 3.0 μg/g fresh weight. Results of uredospore germination test are presented, showing that coumarin concentration in the leaf and pod are below the threshold necessary to stimulate uredospore germination.

INTRODUCTION

Most cultivars of snap beans (*Phaseolus vulgaris* L.) are susceptible to a rust disease incited by *Uromyces appendiculatus* (Pers.) Unger var. appendiculatus. It it well-known that fungal uredospores of dense populations germinate at a very reduced rate or not at all. Inhibition of uredospores germination was believed to be due to chemical constituents, either endogenous or exogenous. Water extracts of *Puccinia graminis* f. sp. *tritici* (wheat rust) demonstrated the presence of this inhibiting component of germination (Allen, 1955), which was later identified from bean rust uredospores as methyl 3,4-dimethoxycinnamate (Macko et al., 1970). Allen (1972) determined that only *cis*-methyl 3,4-dimethoxycinnamate would inhibit the germination of the uredospores, suggesting stereochemical specificity. Investigations of uredospore germination and the inhibitor have been reviewed by Allen and Dunkle (1971), Macko et al. (1976), Allen (1976), and Staples and Yaniv (1976).

Unwashed bean rust uredospores floated on the surface of distilled water had only 0.2% germination while those placed on aqueous leachate from snap bean leaves had 26%. Uredospores washed with distilled water and then placed on the aqueous leachate had over 70% germination (Thomas and Meiners, 1977). Increased germination of the unwashed uredospores placed on the snap bean leachate compared to the uredospores placed on distilled water indicates that one or more biologically active chemical components are present either in or on snap bean leaves. These active chemical compounds are capable of counteracting the effect of the germination inhibitor in uredospores, thus increasing uredospore germination. Studies on *Uromyces phaseoli* (bean rust) showed that chemical structures such as linear, branched, saturated, and unsaturated methyl ketones of six to nine carbons and cyclic ketones greatly stimulate uredospore germination (French et al., 1977). An investigation by van Sumere et al. (1957) demonstrated that coumarin extracted from wheat rust uredosphores at concentrations as low as 10 μg/mL was extremely effective in promoting the germination of uredospores of *P. graminis* f. sp. *tritici*.

Preliminary experiments conducted on the aqueous leachate from snap bean leaves that had increased uredospore germination showed that coumarin was not present at a detectable level. Coumarin was also below the detectable level in condensate water collected from snap bean leaves grown in a dew chamber (Thomas, unpublished data).

As far as we determined in the literature, coumarin has not been isolated from snap bean tissue. Coumarin has been reported to be a hepatotoxic compound in test animals and is listed as a suspected carcinogenic compound (Dickens and Jones, 1965; Cohen, 1979; Evans et al., 1979). Due to the ability of coumarin to counteract the germination inhibitor of uredospores from wheat rust and its importance as a natural toxin in plants, a study was conducted to determine whether coumarin is present in leaf and pod tissue of snap beans and to determine the coumarin concentration required to stimulate maximum bean rust uredospore germination.

MATERIALS AND METHODS

Plant Samples. Five cultivars of snap beans, Eagle, Topcrop, Tender Crop, Mountaineer White Half Runner, and Bush BlueLake 274, were grown at Beltsville, MD. All of the cultivars were susceptible in the field and greenhouse

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