The Effect of Ammoniation Upon Ricinine in Castor Meal

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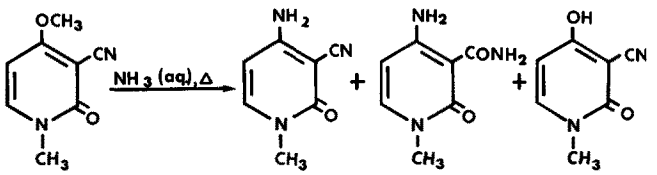
ABSTRACT

When castor meal is detoxified by treatment with ammonia to destroy ricin and various allergens the amount of ricinine, a toxic pyridone normally present in castor, is also reduced by about 25%. It was shown that the majority of ricine loss can be accounted for by the production of 4-amino-3-cyano-N-methyl-2-pyridone, a product formed by displacement of the original methoxyl group of ricinine with ammonia. In vitro enzymatic studies have demonstrated that neither this compound nor ricinine significantly affects lactate dehydrogenase. Another possible derivative of ricinine formed from ammoniation, 4-amino-3-carboxamido-N-methyl-2-pyridone, which was not found in ammoniated castor meal, *did* inhibit lactate dehydrogenase.

INTRODUCTION

Castor pomace, the product remaining after expression of castor oil from the seed, contains 30-36% protein (1) and should therefore be suitable as an animal feed. Nevertheless extensive use of this material has not taken place because of its content of the toxic protein, ricin (2), a highly effective allergen (3-5) and a moderately toxic alkaloid, ricinine (I), the structure of which corresponds to 3-cyano-4-methoxy-N-methyl-2-pyridone (6). Several methods for detoxification of castor meal have been proposed (1,7), one of which (7) involves treatment of the meal with ammonium hydroxide at elevated temperatures. Treated pomace is nonallergenic and no longer contains active ricin; also we have observed that the ricinine content of such meal is reduced. In an effort to determine why this loss of ricinine occurs we treated pure ricinine with aqueous ammonia (under conditions considerably more severe than those employed in meal treatment). Three products were identified as shown in the equation below: an amino-nitrile (II), an



I II III IV

amino-amide (III) and ricininic acid (IV). Of these materials only the amino-nitrile (II) and ricininic acid were found to be present in ammoniated pomace. Syntheses, characterization and properties of these products are reported in this communication including a study of inhibitor effect upon one enzyme system.

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TABLE I

Inhibition of Lactate Dehydrogenase

Added compound, mg/ml	Activity, %
None	100
Ricinine	
0.33	105
Amino amide derivative (III)	
0.045	35
0.09	6
0.18	2
Amino nitrile derivative (II)	
0.28	96

EXPERIMENTAL PROCEDURES

Syntheses

Ricinine (I): Into a 250 ml flask equipped with stirrer, condenser and drying tube were placed 150 ml dry, freshly distilled dimethyl sulfoxide (DMSO) and 33.9 g (197 mmoles) sodium ricininate prepared by the method of Schroeter et al. (8). When solution was complete, methyl iodide, 25 ml (57 g, 400 mmoles), was added in one portion after which the mixture was allowed to stir 2 hr at room temperature. The solution was then poured, with stirring, into 500 ml ether to give a sticky precipitate which was separated from the majority of DMSO by decantation of the etheral mixture followed by washing with an additional 300 ml portion of ether. The remaining semisolid material was taken up in 200 ml hot water; after the pH was adjusted to 11 with solid sodium carbonate, the solution was treated with activated charcoal and filtered. Upon storage overnight at 5 C, crystals formed which were collected by suction and washed successively with 150 ml cold water, 250 ml 95% ethanol and 150 ml ether. After drying in vacuum overnight at room temperature, 21.2 g (129 mmoles, 65.6%) ricinine, mp 198-201 C (lit. mp = 199-201 C [8]) was obtained. The NMR spectrum of ricinine (DMSO-D_6) shows the following signals: δ 3.45 (*s*, 3H N-CH_3), 3.99 (*s*, 3H, O-CH_3), 6.42 (*d*, J = 8Hz, 1H, ring β), and 8.08 (*d*, J = 8Hz, 1H ring α). The IR spectrum (KBr) shows absorptions at 2220 (-CH), 1640 (α-pyridone) and 1600 cm^{-1} (C = C, ring). The fluorescence spectrum (H_2O) shows an excitation peak at 308 nm and an emission peak at 366 nm.

Acidification of the aqueous residue gave, upon filtration and drying, 6.2 g (41 mmoles, 21%) recovered ricininic acid.

4-Amino-3-cyano-N-methyl-2-pyridone (II): Into a 300 ml stainless steel rocking bomb (American Instrument Co.) were placed 11.1 g (67.5 mmoles ricinine and 150 ml dry methanol containing 24 g (1.4 moles) anhydrous ammonia. The mixture was first warmed to 230 C with agitation over a 2 hr period and then cooled to room temperature. The crystals which had separated from solution were collected, and the methanolic filtrate was concentrated to 100 ml, stored at 5 C overnight then filtered to give an additional crop of product. The total weight of amino-nitrile was 6.85 g (46.0 mmoles, 68%). Crystallization from water, 50 ml/g, gave material of mp 249-251 C. Analysis calculated for $C_7H_7N_3O$: C, 56.37; H, 4.73; N, 28.17. Found: C, 56.4, H, 4.68; N, 27.8.

The NMR spectrum of II (DMSO-D_6) consisted of signals at δ 3.28 (*s*, 3H, N-CH_3), 5.81 (*d*, J = 8Hz, 1H, ring β), 7.17 (broad *s*, 2H, -NH_2) and 7.53 (*d*, J = 8Hz, 1H, ring α). Its IR spectrum (KBr) showed absorptions at 3200-3400 (-NH_2), 2220 (-CN), 1660 (α-pyridone) and 1600 cm^{-1} (C = C, ring). The mass spectrum, demonstrating a parent ion at m/e = 149, was consistent with the result of elemental analysis. UV max: (H_2O), 282 nm (ϵ = 1.5 x 10^4). The fluorescence spectrum exhibited an excitation peak at 291 nm and an emission peak at 385 nm in 5.86 M H_3PO_4. In water the excitation peak was at 290 nm and the emission at 258 nm.

TABLE II

Fluorescence Properties of Ricinine and Its Derivatives

Compound	Fluorescence intensity at λ max arbitrary units	λ Max (nm)	
		Excitation	Emission
Ricinine (I)			
In H_2O	67.5	308-310	366
In 2 N HCl	67.5	308-310	366
Amino nitrile (II)			
In H_2O	20	290-293	360
In 2 N HCl	38	290-293	388-390
In 0.95 M H_3PO_4	157	290-293	388-390
Amino-amide (III)			
In H_2O	3	---	360
In 2 N HCl	57	290-292	395
In 0.98 M H_3PO_4	87	288-290	390
Ricininic acid (IV)			
In H_2O	20.7	316-318	350 and 363
In 0.048 N HCl	40.5	303	360
In 0.1 N NaOH	0	---	---

4-Amino-3-carboxamido-N-methyl-2-pyridone (III): Into a 100 ml flask equipped with stirrer and condenser were placed 35 ml water, 2.0 g moist IRA-400 ion exchange resin in the hydroxide form (9), and 0.50 g (3.35 mmoles) II from the above preparation. After refluxing with stirring for 4 days, the hydrolysis was judged to be essentially complete by thin layer chromatography (TLC) (vide infra). The ion exchange resin was removed by filtration with the aid of Celite to entrain fine particles. Evaporation of the filtrate then afforded 0.50 g solid. Crystallization from water, 5 ml, gave 0.26 g (1.56 mmoles, 46%) nearly pure amide, which upon further crystallization from 10 ml of water exhibited a mp of 219-222 C. Analysis calculated for $C_7H_9N_3O_2$: C, 50.30; H, 5.43; N, 25.14. Found: C, 50.5; H, 5.23; N, 25.2.

The NMR spectrum (DMSO-D_6) consisted of signals at δ = 3.32 (*s*, 3H, N-CH_3), 5.85 (*d*, J = 8Hz, 1Hz, ring β), 6.7-7.4 (*d*, broad, 2H, -NH_2), 7.36 (*d*, J = 8Hz, 1H ring α) and 10.0 (*s*, broad, 2H -$CONH_2$). Its IR spectrum ($CHCl_3$) showed absorptions at 3500 and 3300 (NH_2 and $CONH_2$) and at 1660 cm^{-1} (-$CONH_2$ and α-pyridone) with absence of absorption in the 2200 cm^{-1} region. The mass spectrum gave rise to a parent ion at m/e = 167, consistent with the proposed formula. The uv spectrum (H_2O) demonstrated a maximum at 285 nm (ϵ = 1.0 x 10^4). Fluorescence spectrum (2*N* NCl) showed an excitation peak at 292 nm and an emission peak at 395 nm.

Thin Layer Chromatography

Eastman Chromogram sheets of silica gel (No. 6060) or alumina (No. 6063) containing fluorescent indicator were used. Two solvent systems were employed and were composed on a volume basis as follows: solvent A: toluene-isopropanol-acetic acid-water; 10:5:1:1; and solvent B: 1,2-dichloroethane-acetonitrile; 1:4. After development of the chromatogram, visualization of spots was accomplished by illuminating the strip with short wavelength (254 nm) UV light whereby compounds of interest became visible as dark spots on an orange background. Additionally it was found that spraying the developed chromatogram with 5.86 M phosphoric acid caused the amino-nitrile (II) to exhibit characteristic violet fluorescence under 254 nm irradiation.

Electrophoretic separations were also accomplished on silica gel chromogram sheets in a solution of 0.2 M NaH_2PO_4. Thus ricininic acid could be separated and visualized as above.

Ammonia Treatment

In a preliminary experiment 2 g ricinine and 80 ml 8 M aqueous ammonia were placed in a stainless steel bomb and heated to 150-160 C for 19 hr. Upon analysis by TLC and isolation by fractional crystallization of products formed, it was observed that both the amino-nitrile (II) and amino-amide (III) were formed, and that all ricinine was consumed.

Ammoniated pomace consisted of castor meal treated at 80 C in a pressure tight container with 6 M ammonium hydroxide for 45 min (7).

Extraction and Analysis of Castor Meal

Into a Soxhlet extractor were placed 12.5 g ground castor meal, and extraction with methanol was effected over an 8-13 hr period. After evaporation to dryness the extract was taken up in 13 ml hot water, cooled to room

TABLE III

R_f Values of Ricinine, Its Derivatives, and Castor Meal Extracts[a]

Material or compound	R_f		
	Solvent A		Solvent B
	Silica gel	Alumina	Silica gel
Ricinine	0.19	0.40	0.73
Ricininic acid	0.07	0.02	0.01
Amino nitrile	0.12	0.19	0.63
Amino amide	0.27	0.33	0.42
Untreated meal extract			
Presumed ricinine spot	0.20	0.38	0.72
Ammoniated meal extract			
Presumed ricinine spot	0.21	0.43	0.80
Presumed amino nitrile	0.14	0.23	0.66

[a]In a separate experiment the presumed spots cochromatographed with the authentic compounds they were presumed to be.

TABLE IV

Analysis of Castor Meal Extracts

Component analyzed	Analysis[a], mmoles/100g	
	Untreated meal	Ammoniated meal
Ricinine (I)	1.42	1.08
Amino-nitrile (II)	0	0.22
Ricininic acid (IV)	0	ca. 0.013[b]
Total	1.42	1.31

[a]Analyses were performed by fluorometric determination on material obtained from thin layer chromatography separations.

[b]Approximate analysis was obtained on material eluted from thin layer electrophoresis separations.

temperature and washed with four 10 ml portions of ether. The ether extract was discarded, and the remaining aqueous suspension was evaporated to dryness. The gummy residue was repetitively triturated with acetonitrile until examination by TLC showed that no ricinine or ricinine-derived material remained.

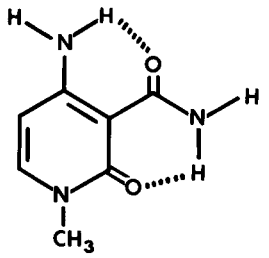
Fluorometric analyses of these compounds were accomplished with a G.K. Turner Model 210 Spectrophotofluorimeter. Ricinine was analyzed in 0.083 N NaOH solution using an excitation wavelength of 320 nm. The difference between fluorescence emission at 365 nm and at 445 nm was determined and was correlated with the amount of ricinine present by use of a standard curve. Analysis of amino-nitrile (II) was done in 4.88 M H_3PO_4 using an excitation wavelength of 270 nm. The quantity present was determined from the difference between the emission at 390 nm and 365 nm. The excitation wavelengths for these analyses was deliberately displaced from the maxima (308 nm for ricinine and 295 nm for II) to minimize interference from the fluorescence of contaminants. Error from light scattering was minimized by taking the difference between two emission wavelengths to subtract out baseline variation.

Ricininic acid in 0.093 N NaOH solution was excited at 320 nm. The difference in fluorescence intensity at 350 nm before and after addition of H_3PO_4 to a concentration of 0.39 M was used to determine the amount present.

In the study of lactate dehydrogenase (L-lactate-NAD oxidoreductase, E.C. No. 1.1.1.27) inhibition, the rate of NADH consumption was measured by observation of UV absorption at 340 nm. Because ricinine (at the concentrations used in these experiments) absorbs strongly at 340 nm, NADH oxidation had to be observed at 345 nm when this compound was used. Control experiments without ricinine were also determined at 345 nm so that comparison of activities with and without ricinine would be valid. The reaction mixtures (3 ml in volume) were 3.3 x 10^{-3} M in lithium pyruvate and contained 0.2 mg NADH in 0.33 M phosphate buffer (pH 7). Inhibitors were added from stock solutions to make up the concentrations shown in Table I, and the reactions were initiated by addition of the enzyme (0.096-0.144 I.U.B. units).

RESULTS AND DISCUSSION

The proposed structures II and III are consistent with the data obtained through IR, NMR, mass spectrometry and chemical analysis. The IR bands expected for amino and nitrile or amide groups are present in the appropriate compound while the respective NMR spectra confirm that ring substitution pattern remains unchanged. The amino-amide most likely exists in the strongly internally hydrogen-bonded form V, below. Several factors support this structural arrangement: the lower melting point with respect to the amino-nitrile (ca. 222 vs. 251 C for the nitrile II) suggests that intermolecular association is reduced in contrast to the situation usually experienced with amides (cf. nicotinamide, mp 129-131 C vs. nicotinonitrile, mp 50-51 C). Also the solubility in relatively nonpolar organic solvents was greater for the amide than for the nitrile. The NMR spectrum of the amino-amide shows broadening and splitting of the amino-proton peaks and a signal of two rather acidic protons appears at δ = 10.0 ppm. These observations may be explained by the intramolecularly hydrogen bonded structure V.



V

Both the UV absorption and the fluorescence properties of these two compounds are similar. Acidification of their solutions enhances the fluorescence intensities with that of the amide increasing 20-fold and that of the amino-nitrile by a factor of two in 2 N HCl (Table II). Increase of fluorescence is somewhat more pronounced in phosphoric acid solution. In acidified media the excitation wavelengths remained stationary while emission maxima shifted about 30 nm toward the red. The fluorescence characteristics can be used to distinguish the two compounds from ricinine whose intensity, excitation wavelength and emission peaks are invarient upon acidification. Ricininic acid (IV) undergoes approximate doubling of fluorescence intensity in acidified solution while basic solutions exhibit no fluorescence. These properties have been utilized in the development of analytical procedures described in the experimental section.

Of the two amino-bearing compounds, the amide (III) is more likely to interfere with the activity of pyridine nucleotide containing enzyme systems, a possible mode of toxicity. As seen from Table I, it inhibited the activity of lactate dehydrogenase, an enzyme widely distributed in heart, liver and muscle tissues, almost completely at concentrations where the nitrile (II) or ricinine itself had no significant effect. Significantly however the amino-amide is not found in ammoniated castor meal. Its absence may be inferred from the fact that no spot corresponding to this material was found on TLC of extracts employing either of the two solvent systems with both adsorbents used.

Extracts of both nontreated and ammonium hydroxide-treated castor meal (same original lot) were shown to contain ricinine which in the raw meal amounted to about 0.23% in reasonable agreement with reported values (10). Ricinine in the extracts was identified by cochromatography with authentic material using solvent A with both silica gel and alumina adsorbents and with solvent B using silica gel according to the procedure described for TLC. Table III expresses these results. In addition the fluorescent properties of ricinine extracted from meal were identical to those of the standard. As judged from the results of TLC experiments, no amino-nitrile was present in the raw meal. Extracts of ammoniated pomace, however, contained this material (Table IV). Identification of this compound as II was based on both its chromatographic behavior and the observed fluorescence properties of material obtained by elution of the appropriate spot on the chromatogram.

Electrophoretic examination of meal extracts revealed that although material from both treated and nontreated sources showed substances migrating similarly to authentic ricininic acid, only the latter extract contained that material. Fluorescence measurements in acid solution on

material isolated from the TLC strip confirmed the presence of ricininic acid in that case. The amount of this material was estimated to be less than 1% of total ricinine initially present.

Approximately 16% of the ricinine in castor meal was converted to the amino-nitrile derivative during ammonia treatment with about 92% of starting ricinine accounted for. It is evident from the results of these experiments that all ricinine present in the meal could be completely destroyed by prolonged treatment or by use of higher temperature. However it cannot be assumed that even this would render the meal innocuous, as it remains to be demonstrated that the ricinine-derived material is less toxic than is ricinine. Experiments are currently underway in our laboratory to evaluate the effects of the amino-nitrile (II) upon whole organisms.

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