a value of 3.6 ± 0.7 kcal/mol is obtained. Considering the results involving the single-chain inhibitor in cacodylate and PIPES buffers and that cacodylic acid and PIPES have rather different values for $\Delta H_{ionization}$ (0.3 and −3 kcal/mol, respectively, under our conditions), we further conclude that essentially no protons were taken up by the buffer when trypsin reacted with this form of the inhibitor.

The most recent and thorough calorimetric investigations of the reactions of trypsin inhibitors with trypsin were with the Bowman–Birk inhibitor (Turner et al., 1975) and the Kunitz inhibitor (Barnhill and Trowbridge, 1975) from soybeans. Corn trypsin inhibitor is quite different structurally from both of these proteins. The Bowman–Birk inhibitor has a molecular weight of 7000 and inhibits chymotrypsin as well as trypsin. The corn inhibitor has a molecular weight of 12 500 and does not inhibit chymotrypsin (Swartz et al., 1977). The Kunitz inhibitor, on the other hand, is much larger than the corn trypsin inhibitor, and these two proteins are not immunologically cross reactive (R. S. Corfman and G. R. Reeck, unpublished results, 1977). Furthermore, the corn inhibitor is distinct in having four tryptophan residues (Swartz et al., 1977). A comparison of our results with those reported in the earlier studies is therefore of interest.

Our results agree qualitatively with both those of Turner et al. (1975) and Barnhill and Trowbridge (1975) whose data indicate that at pH 6.5 the association of each soybean inhibitor with trypsin should be favorable thermodynamically with unfavorable enthalpy changes being overcome by favorable entropy changes. Our data lead to the same conclusions for corn trypsin inhibitor. A closer look at certain aspects of the assembled data, however, suggests a difference that is considered worthy of further investigation.

The data for the association of soybean trypsin inhibitor with trypsin which have been provided by Turner et al. do not include the pH we used but those provided by Barnhill and Trowbridge do. Accordingly one can interpolate the latter data to estimate enthalpy changes that would be expected to occur at our pH. When this is done it appears our enthalpy changes are significantly larger than those for the Kunitz inhibitor. Further, this approach suggests that the enthalpy changes for the association reactions of bovine β-trypsin with Kunitz soybean inhibitor and the inhibitor in which the Arg-63-Ile bond has been cleaved should be about 0 and 2 kcal/mol, respectively, at pH 6.5. If this is true the enthalpy change for the conversion of virgin to cleaved Kunitz inhibitors should be about −2 kcal/mol under these conditions. This is in good agreement with calorimetric data on peptide bond hydrolysis taken in Sturtevant's laboratory (Rawitscher et al., 1961) which suggest that ΔH for the hydrolytic reaction involved in converting a single- to a two-chain inhibitor should lie in the range of −1 to −2 kcal/mol. In contrast, our enthalpy data indicate that the conversion of single- to two-chain corn trypsin inhibitor is near zero and suggest that this conversion involves processes other than a simple hydrolysis of a peptide bond. For instance there might be a conformational change and/or changes in solvent interactions.

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Thin-Layer Chromatographic Analysis of Nitrofurans in Feed Premixes

A simple method for the assay of furazolidone and furaltadone, substances frequently associated for the prevention of coccidiosis in veterinary medicine, is described. A chromatographic separation technique was carried out before spectrophotometric assay for isolating these nitrofurans from complex pharmaceutical preparations such as feed premixes. The study of the different factors likely to influence recovery and assay of furazolidone and furaltadone from such products allowed an accurate and reproducible quantitative analysis.

Some substances resulting from a reaction of the 5-nitro-2-furaldehyde with different hydrazine groups are frequently used in veterinary medicine for their antimicrobial and anticoccidial activities. These nitro drugs are often blended with other antimicrobial drugs and growth factors in cereal meals; the so obtained premixes are generally in the form of more or less homogeneous powder. Mixed with the normal feed of the animals, generally at low levels (50–200 ppm), these drugs are used in both poultry and swine husbandry for the prevention of enteritis, coccidiosis, and salmonellosis.

Several methods have been reported for the analysis of these compounds. Most of them involved spectrophotometric assays after conversion of the nitrofurans to their

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phenylhydrazones, like the current official AOAC method (1975). They were applied to analysis from blood (Buzard et al., 1956), tissues (Herret and Buzard, 1960), milk (Cox and Heotis, 1963; Stone, 1964), and drugs (Cross et al., 1960). More recently some authors described different methods involving microbiological assays (Gang and Shaikh, 1972), gas–liquid chromatography (Ryan et al., 1975), column or thin-layer chromatographic separation followed by spectrophotometric assays (Wojtowicz and Diliberto, 1971; Zoni and Lauria, 1967; Bortoletti and Perlotto, 1968). However, the quantitative analysis of pharmaceutical preparations containing several nitrofurans, the physicochemical properties of which are very similar, has not yet been reported by these methods. Now, the association of nitrofurans in drugs, which is frequent in veterinary medicine, brings up a real problem for quality control of finished pharmaceutical products.

Therefore our laboratory attempted to carry out a quantitative analysis of nitrofurans often associated in complex veterinary preparations such as feed premixes: furazolidone [3-(5-nitro-2-furfurylideneamino)-2-oxazolidinone] and furaltadone [5-morpholinomethyl-3-(5-nitro-2-furfurylideneamino)-2-oxazolidinone].

After solubilization, the nitrofurans were separated by thin-layer chromatography and then extracted before spectrophotometric quantitative determination. The chromatographic separation, necessary because of the similarity between the absorption spectra of the two nitrofurans, also allowed the elimination of some substances such as vitamin A which may interfere in the spectrophotometric assay.

EXPERIMENTAL SECTION

Apparatus and Samples. *N,N*-Dimethylformamide (DMF) used for the solubilization of the nitrofurans, chloroform, and acetone used as chromatographic solvents, and silica gel precoated TLC plates (20 × 20 cm; layer thickness, 0.5 mm) with fluorescent indicator were purchased from Merck (Darmstadt, West Germany). Standards of furazolidone and furaltadone were graciously supplied by Vetoquinol S.A., Lure, France. Instruments included a Beckman Acta III spectrophotometer.

Extraction. In a screw-capped vial, accurately weigh a sample of the feed containing about 2 mg of each nitrofuran. Add 10 mL of dimethylformamide (DMF) and stopper the vial. Extract gently by means of a magnetic stirrer for 30 min. Transfer in a 15-mL tube and centrifuge at 4000 rpm for 5 min.

Chromatographic Separation. The supernatant solution (150 μL) is applied on the plates as a continuous line of 15 cm long and less than 3 mm wide. Allow the chromatographic development in the dark with chloroform/acetone (70:30) until the front of solvent is at about 15 cm from the spot straight (45 min at 25 °C).

After air-drying, quickly locate and mark out the bands of furazolidone (R_f 0.40) and furaltadone (R_f 0.13) by means of UV light (254 nm). The exposure of the plates to UV light should be as short as possible (a few seconds) because of the high photosensitivity of the nitrofurans. Scrape off these bands and transfer silica gel into screw-capped vials. Add 10 mL of DMF and submit the stoppered vials to extraction on a magnetic stirrer for 10 min. Transfer to a 15-mL tube and centrifuge for 15 min at 5000 rpm.

Quantitative Spectrophotometric Analysis. Carefully pipet the supernatant extract containing about 3 μg of nitrofuran/mL and determine the absorbance at 370 nm against a DMF blank. Determine the amounts of furazolidone and furaltadone of the extracts from standard curves established for each nitrofuran as follows.

Table I. R_f Values for Thin-Layer Chromatograms of Furazolidone and Furaltadone with Different Development Systems

	development systems[a]		
	1	2	3
furazolidone	0.40	0.21	0.30
furaltadone	0.13	0.04	0.26

[a] 1, chloroform/acetone (70:30); 2, 1-butanol/diethyl ether/acetone (10:85:5); Zoni and Lauria (1967) 3, dioxan/benzene (50:50), Bortoletti and Perlotto (1968).

Table II. Absorbances at 370 nm of Furazolidone and Furaltadone in 10 mL of DMF

concn, μg/10 mL of DMF	mean absorbance ± SD[a]	
	furazolidone	furaltadone
10	0.074 ± 0.003	0.052 ± 0.001
25	0.186 ± 0.006	0.132 ± 0.002
50	0.379 ± 0.014	0.266 ± 0.006
75	0.572 ± 0.014	0.402 ± 0.008
100	0.755 ± 0.021	0.537 ± 0.013

[a] Mean of four analysis.

Accurately weigh 100 mg of furazolidone and furaltadone standards into 100-mL volumetric flasks and dissolve in and dilute to volume with DMF. From each solution, transfer 10, 25, 50, 75, and 100 μL into 10-mL volumetric flasks by means of micropipets (Pedersen) and dilute to volume with DMF. The absorbance of these solutions was determined in the same conditions as above.

The amounts of nitrofurans present in the initial sample of drug (a) were calculated by the following equation: $a = b \times 10/0.15$, where b is the value found in the quantitative assay.

Make sure of the purity of extracted nitrofurans by comparison of their spectra with standard solutions.

Protect nitrofuran solutions from sunlight at all times to avoid decomposition.

RESULTS AND DISCUSSION

Separation of Furazolidone and Furaltadone by Thin-Layer Chromatography. The developing solution used, chloroform/acetone, gave satisfactory results, and the ratio 70:30 appeared the best for a minimum diffusion of the spots and a good separation of furaltadone from starting line to avoid contamination by any nonrunning substance.

Development solvents of Zoni and Lauria (1967) and of Bortoletti and Perlotto (1968) could not be used here because the R_f values of furaltadone are respectively too low or too close to the R_f value of furazolidone (Table I).

Moreover, furazolidone and furaltadone applied as spots in a concentration range between 0.4 and 40 μg/10 μL showed that the amounts of nitrofuran do not affect the R_f values.

Nitrofurans Assays in Feed Premixes. The spectrophotometric analysis of furaltadone and furazolidone at 370 nm gave standard curves with a good linearity between selected values and reproducibility within narrow limits (Table II).

Some factors likely to interfere in the assays were submitted to the following studies.

The extraction rate of nitrofurans from commercial premixes was determined with stirring times of 15, 30, 60, and 120 min; entire extraction was obtained within 30 min. Moreover, a stirring time of 30 min with 10 mL of DMF of nitrofuran-free commercial premixes, in which we added

Table III. Recoveries of Furazolidone and Furaltadone from a Premix Fortified with Graded Amounts of Nitrofurans

concn, mg/g	recovery, %	
	furazolidone	furaltadone
1	100	111
2	96	101
3	99	102
4	96	96

furazolidone and furaltadone at 1, 2, 3, and 4 mg/g, gave recovery percentages compiled in Table III. It appears that nitrofuran extraction from premixes was complete in such experimental conditions.

Extraction tests of furazolidone and furaltadone, from silica gel of the chromatoplates, were realized with stirring times from 5 to 30 min in the DMF. The results showed the extraction of these nitrofurans was achieved within 10 min and the recovery was entire.

Because of the well-established sensibility of nitrofurans to light, a study of the decomposition of these products in DMF was carried out. Degradation rates of furazolidone and furaltadone after sunlight exposures of 5, 15, and 30 min, respectively, reached the values of 18, 36, and 42%.

The extraction of nitrofurans from feed premixes was complete in the precited conditions. An evaluation of the losses which may happen after this extraction was attempted; four samples containing a mixture of 2 mg of each nitrofuran in 10 mL of DMF were treated according to the above mentioned conditions. These assays allowed recoveries of 98.3% for furaltadone and 95% for furazolidone. The so determined small losses were probably due to light decomposition which cannot be completely avoided.

This assay method of furaltadone and furazolidone was applied to the analysis of commercial feed premixes. The reproducibility of the results from such products was satisfactory as well as that obtained with premixes in which nitro drugs were added by ourselves. Thus, standard deviations of the results were the equivalent of about 5% of the amount of furaltadone or furazolidone when present at 800 ppm and reduced to about 1.5% when present at 2000 ppm, the mean quantity of nitrofurans in commercial premixes.

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Effect of Vegetable Juices and Milk on Alkylating Activity of *N*-Methyl-*N*-nitrosourea

Effect of vegetable juices and milk, which are regarded to be the low-risk foods for gastric cancer, on the alkylating activity of *N*-methyl-*N*-nitrosourea (MNU) toward 4-(*p*-nitrobenzyl)pyridine was investigated to gain some information about dietary factors for stomach cancer. The juices and milk effectively decomposed MNU and consequently decreased its alkylating activity. The results suggest that they may play an important role to prevent human stomach cancer caused by alkylating agents.

Carcinogenic activity of *N*-nitroso compounds is believed to be due to their alkylating properties toward nucleophilic groups in proteins and nucleic acids (Magee and Barnes, 1967; McCalla, 1968; Swann and Magee, 1968). Among these compounds, alkylnitrosamides, which have powerful carcinogenic activity without metabolic activation, are the most possible carcinogens to cause human stomach cancer (Marquardt et al., 1977). In our previous work we studied the alkylating activity of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) to 4-(*p*-nitrobenzyl)pyridine (NBP) in some vegetable juices and found that they decomposed MNNG to noncarcinogenic *N*-methyl-*N'*-nitroguanidine, suggesting the presence of an anticarcinogenic effect in some fresh vegetables (Yano and Morita, 1977). This result was also in good agreement with earlier findings of the antimutagenic effect of vegetables in other systems (Kada, 1977).

Since a negative correlation between the consumptions of some fresh vegetables (Haenszel et al., 1972) or milk (Hirayama, 1963) and gastric cancer has been reported, it seems to be of interest to study the effect of these foods on the alkylating activity of alkylnitrosamides. Thus I investigated the alkylating activity of *N*-methyl-*N*-nitrosourea (MNU) on NBP in milk and the following vegetable juices: garden pea (*Pisum sativum* L.), tomato (*Lycopersicum esculentum* var. *commune*), celery (*Apium graveolens* var. *dulce*), radish (*Raphanus sativus* var. *hortensis*), lettuce (*Lactuca scariola* var. *sativa*), cucumber (*Cucumis sativus* L.), and cabbage (*Brassica oleracea* var. *capitata* L.). In this communication, the results of the above alkylations as well as some factors which influence the alkylating activity are reported.

METHODS AND MATERIALS

MNU was prepared by the method developed by Arndt (1943) and recrystallized from ether. NBP (Tokyo Kasei Co.) and 3-amino-1-propanol (Tokyo Kasei Co.) were used without purification. Vegetables and milk were purchased

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