only a slight effect on the total renal excretion of radioactivity. When given orally, there was an increase in the 12–24-hr period excretion in the Mixture I and Mixture II groups, but this could have been due to delayed absorption. It is possible that there was reduced excretion during the first few hours which was not apparent by 12 hr when the urine was collected.

The most significant effect of aspirin was on the proportions of the major metabolites of acetaminophen excreted in urine. This effect was seen whether acetaminophen was given orally or intravenously. The principal change was the reduction in both the amount and the proportion of acetaminophen sulfate. The increased proportions of free acetaminophen and the glucuronide and mercapturate metabolites could well be consequences of diminished sulfate conjugation. The reason for this effect of aspirin is not immediately apparent, since salicylate is not known to be conjugated with sulfate; therefore, competition for the available sulfate was not possible. However, Boström *et al.* (12) showed that salicylate inhibits the biosynthesis of mucopolysaccharide sulfates and the excretion of ester sulfates in urine. These investigators suggested that the known oxidative uncoupling action of salicylate reduced the supply of adenosine triphosphate and, hence, active sulfate (3′-phospho-5′-adenosinephosphosulfate), which is necessary for sulfate conjugation. It is possible, therefore, that the reduced amount of acetaminophen sulfate could be due to less active sulfate being available in the aspirin-treated rats.

Levy and Regardh (3) and Amsel and Davison (4) did not see any changes in acetaminophen sulfate in human urine when doses of aspirin in the high therapeutic range were given. This could be due to sulfate conjugation being quantitatively less important in humans than in rats or to the doses being less than abusive. The increased excretion by the aspirin-treated rats of a metabolite assumed to be acetaminophen mercapturate could be toxicologically important since this type of metabolite usually arises from a highly reactive precursor. In humans, both the mercapturate (10) and its immediate precursor, the cysteine conjugate (11), are found in urine after administration of normal doses of acetaminophen. In the rat, very little of the cysteine conjugate is found. If the greater excretion of unchanged acetaminophen in urine represents higher levels in the body, the duration of action of acetaminophen will be longer when aspirin is also administered.

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Field Desorption Mass Spectrometry of Azathioprine and Its Metabolites

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Abstract □ The field desorption mass spectra of azathioprine, I, and some of its metabolites, II–IV, were investigated and compared to their electron impact spectra. The field desorption spectra were shown to be especially well suited for the determination of molecular weights in drug metabolism studies of drugs conjugated with amino acids and peptides. This technique was applied to a metabolite of azathioprine isolated from rat urine and proved to be useful.

Keyphrases □ Azathioprine and metabolites—field desorption mass spectra, compared to electron impact spectra, application to molecular weight determinations □ Mass spectroscopy—comparison of field desorption and electron impact spectra, azathioprine and metabolites, application to molecular weight determinations □ Field desorption mass spectra—azathioprine and metabolites, compared to electron impact spectra, application to molecular weight determinations

Azathioprine[1] (I), [(1-methyl-4-nitroimidazol-5-yl)thio]purine, is the most widely used immunosuppressive agent in clinical organ transplantation. The metabolism of I has been the subject of extensive studies (1–7). When the metabolic fate of the methylnitroimidazole moiety of ^{14}C-azathioprine, labeled in the imidazole ring, was investigated (3, 5, 7), evidence was found that nucleophilic attack occurred on the nitromethylimidazole ring *in vivo*, leading to the formation of 5-substituted derivatives. In recent studies in the rat (3, 6), the following metabolites of azathioprine were characterized: 1-methyl-4-nitro-5-(*S*-glutathionyl)imidazole (II), 1-methyl-4-nitro-5-(*N*-acetyl-*S*-cysteinyl)imidazole (III), and 1-methyl-4-nitro-5-carboxymethylaminoimidazole (IV).

The predominant analytical methods used to iden-

[1] Imuran, Burroughs Wellcome Co.

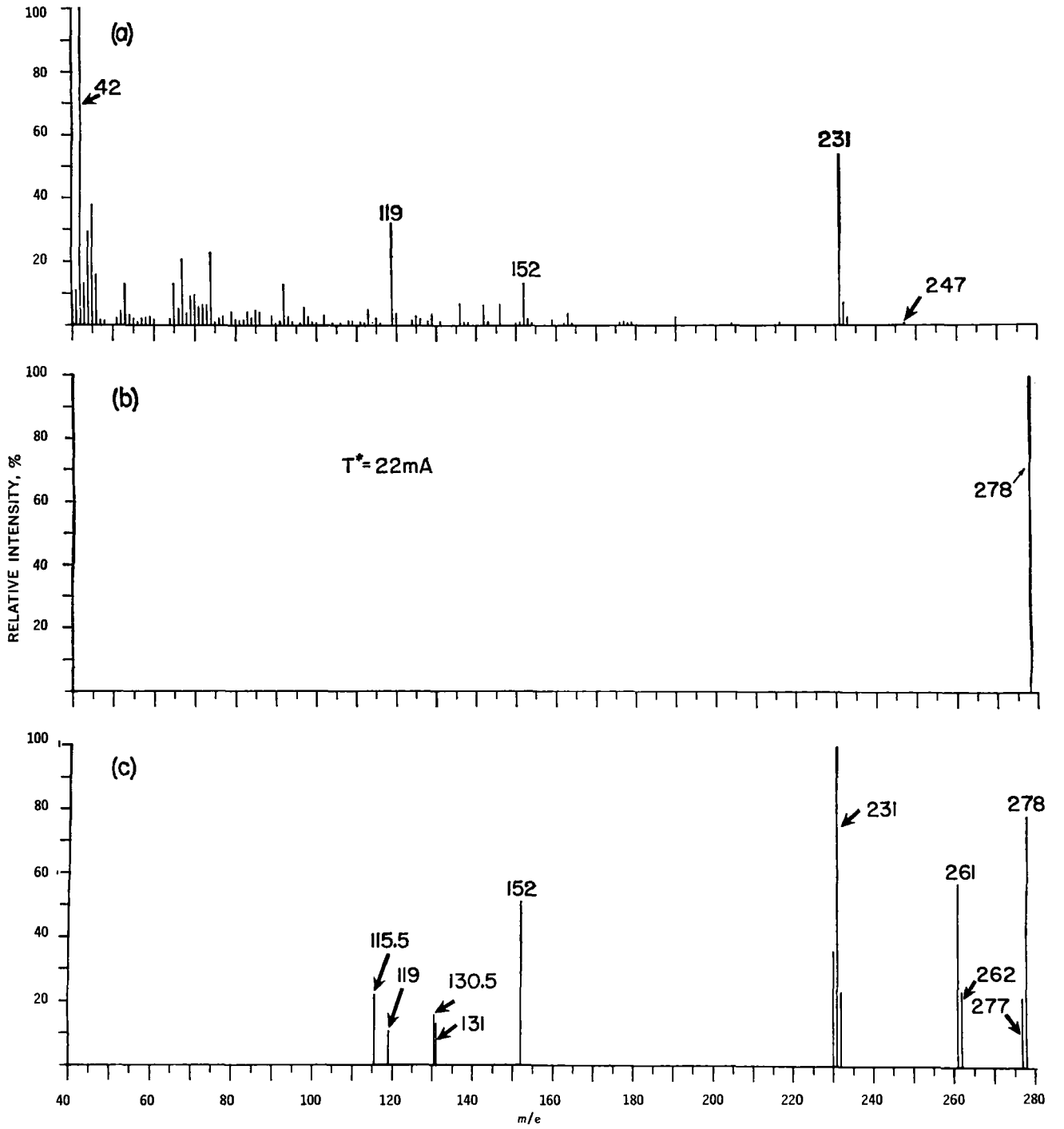


Figure 1—*Mass spectra of azathioprine (I). (a) Electron impact, using a direct probe at a temperature of 210°. (b) Field desorption, using 22 mamp emitter heating current. (c) Field desorption, using 25 mamp emitter heating current.*

tify II–IV were liquid chromatography and UV spectroscopy. Conventional electron impact mass spectrometry was of limited value in the study, because the resulting spectra had small to nonexistent molecular ions and weak high mass fragments. Therefore, molecular weights and/or elemental compositions of these metabolites were difficult or impossible to obtain by electron impact mass spectrometry.

Recent advances in the field of mass spectrometry have yielded techniques that allow the determination of the molecular weight of highly polar compounds whose volatility and/or thermal stability are limited. One such technique is field desorption mass spectrometry (8). Both low- (9–12) and high- (13, 14) resolution spectra can be obtained from polar compounds without derivatization. The observation that unprotected amino acids and oligopeptides exhibit high molecular ion intensities with field desorption mass spectrometry (9, 10) prompted the exploration of this method for the study of metabolites that are conjugates of drugs with amino acids and oligopeptides.

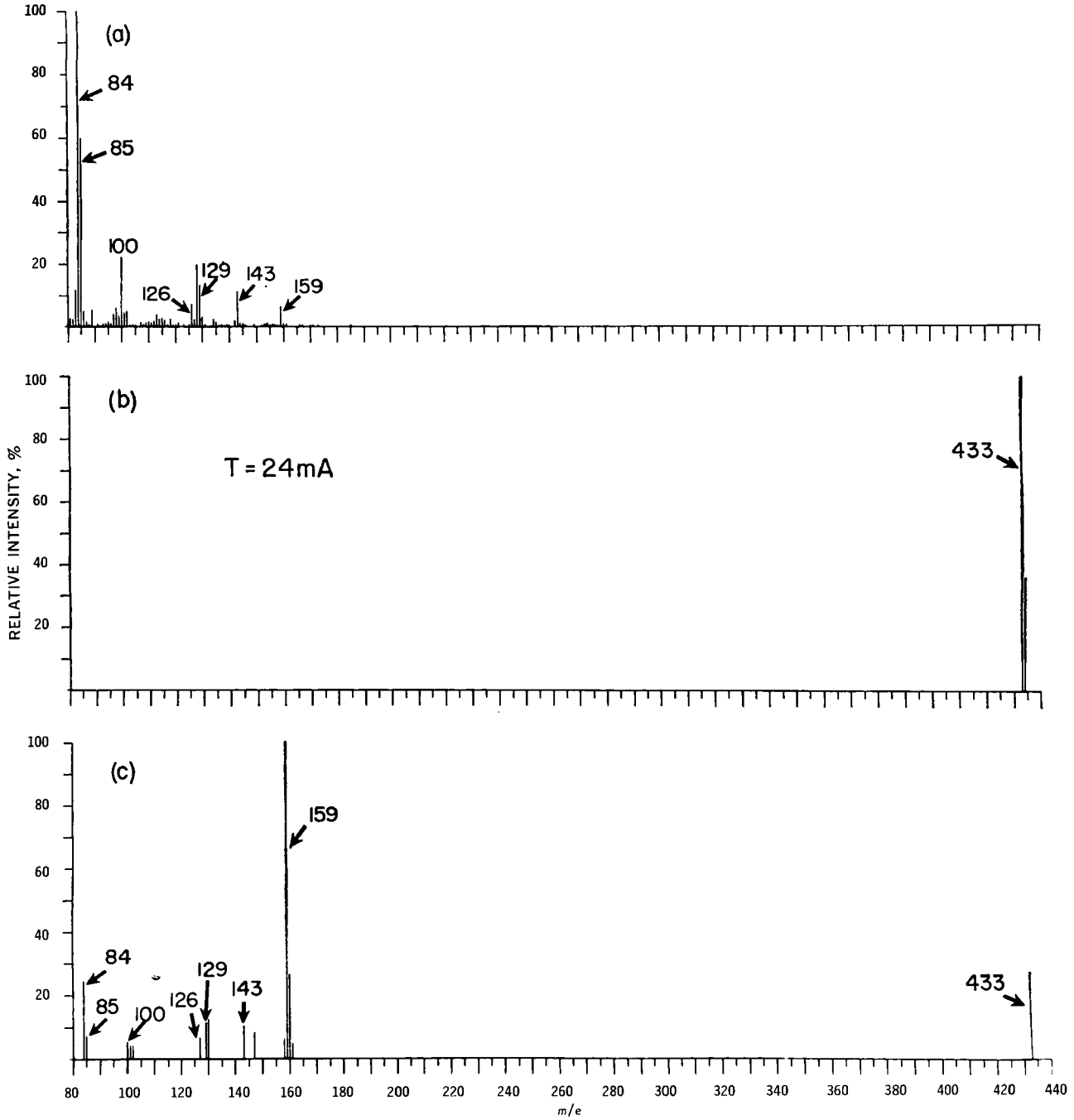


Figure 2—*Mass spectra of II. (a) Electron impact, using a direct probe at a temperature of 195°. (b) Field desorption, using 24 mamp emitter heating current. (c) Field desorption, using 27 mamp emitter heating current.*

EXPERIMENTAL

Compounds I–IV were synthesized[2] (3, 6).

The high-resolution field desorption mass spectra were recorded on a modified double-focusing mass spectrometer[3] equipped with an emitter micromanipulator (14), using vacuum evaporated silver bromide plates[4] for photographic detection. The emitters were 10-μm tungsten wires activated at high temperature (15). The average length of the carbon microneedles produced was about 25 μm. These emitters are of high chemical and mechanical stability; they offer a large adsorption surface and yield outstanding ionization efficiency. The accelerating voltages were +10 kv for the field anode and −2 kv for the slotted cathode plate. The observed total emission measured at the slotted cathode plate was in the range of 10^{-7} amp when optimal emitter heating current was reached. The samples were processed using the emitter dipping technique (8). All samples were adsorbed onto the emitter from aqueous solutions, except I which was applied as a suspension in acetone. The sample consumption was in the order of 5×10^{-8} g of adsorbed material on the emitter surface. For precise mass measurements the photoplates were evaluated by a comparator in connection with a computer[5]. The resolution exceeded 15,000 (10% valley definition) for all spectra. The error in the mass determination was 10 ppm on the average.

[2] At the Wellcome Research Laboratories.

[3] A CEC21-110B double-focusing mass spectrometer was used for accurate mass measurements.

[4] Ionomet, Burlington, Mass.

[5] PDP 8/E.

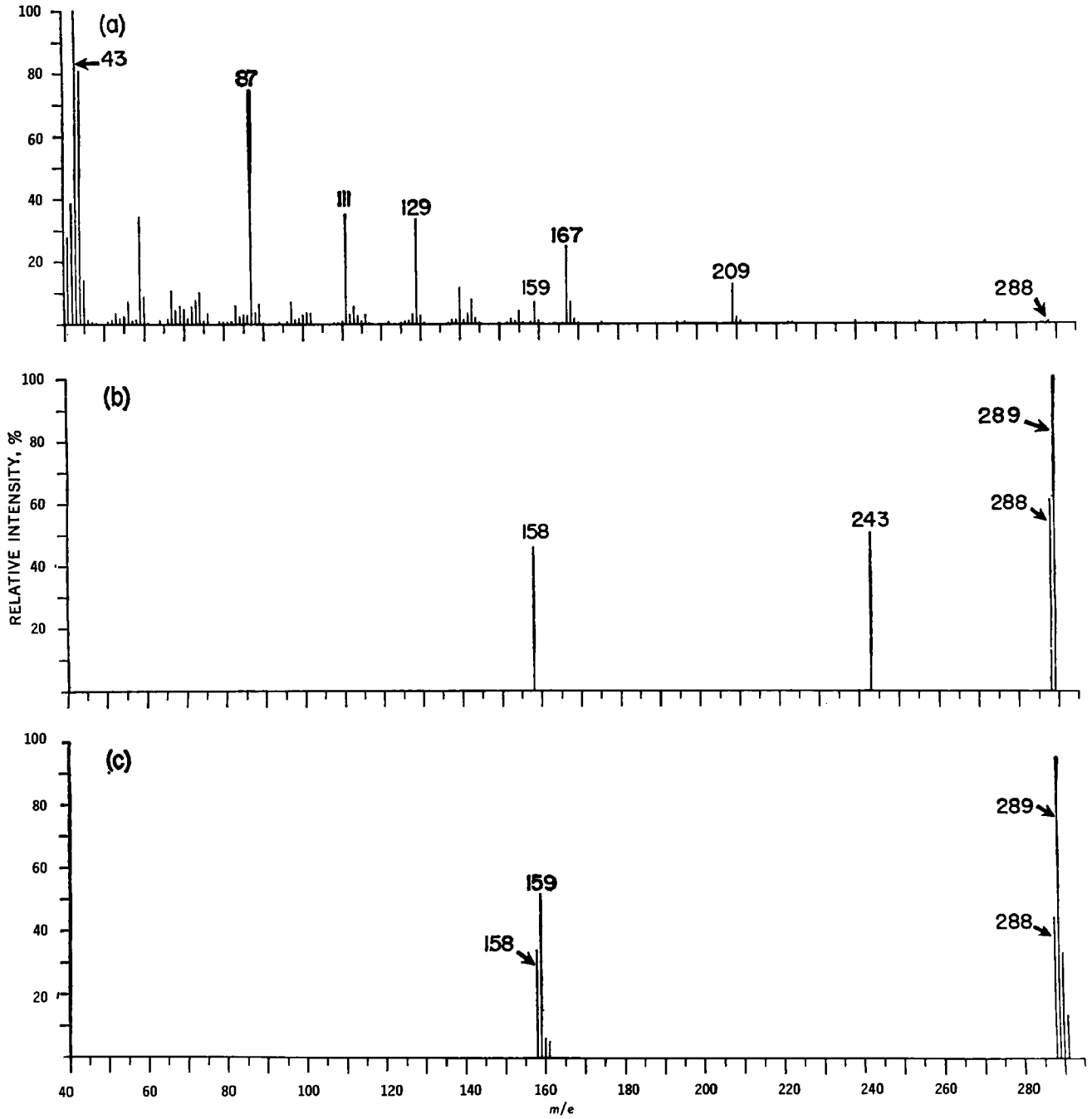


Figure 3—*Mass spectra of III. (a) Electron impact, using a direct probe at a temperature of 150°. (b) Field desorption, using 19 mamp emitter heating current. (c) Field desorption, using 24 mamp emitter heating current.*

The low- and high-resolution electron impact mass spectra were obtained on a second double-focusing mass spectrometer[6] equipped with a data system[6]. Accurate mass measurements were taken by peak matching at 10,000 resolution (10% valley definition). Low-resolution field desorption mass spectra were run on the same system using a source constructed as reported (16). The emitters were also used in the low-resolution work. Accelerating voltages of +3 kv for the field anode and −5 kv for the slotted cathode were applied. A distance of 2 mm was set between the anode and cathode. The secondary electron voltage of the electron multiplier was adjusted to 3 kv. The multiplier used had a gain of 10^6 at 1.7 kv. A scan speed of 2 sec/decade and a computer sampling rate of 6 kHz were selected.

The computer was calibrated by utilizing a previously obtained electron impact calibration and adjusting it to a field ionized spectrum of perfluorotributylamine (V) taken with the field desorption–field ionized source[7]. The field ionized spectrum of V was run at a source pressure of 5×10^{-5} torr and scanned immediately after the emitter was heated, using a current of 50 mamp, and cooled. Under these conditions, 11–22 peaks could be recorded and were used for mass calibration.

RESULTS AND DISCUSSION

Compound I exhibits no M‡ in the electron impact spectrum (Fig. 1a). A small peak at *m/e* 247 ($C_9H_7N_6OS$) represents the loss

[6] Varian Mat CH5DF mass spectrometer equipped with the spectrosystem 100 data system.

[7] The adjustment is accomplished by a program called XCAL contained in the Varian software.

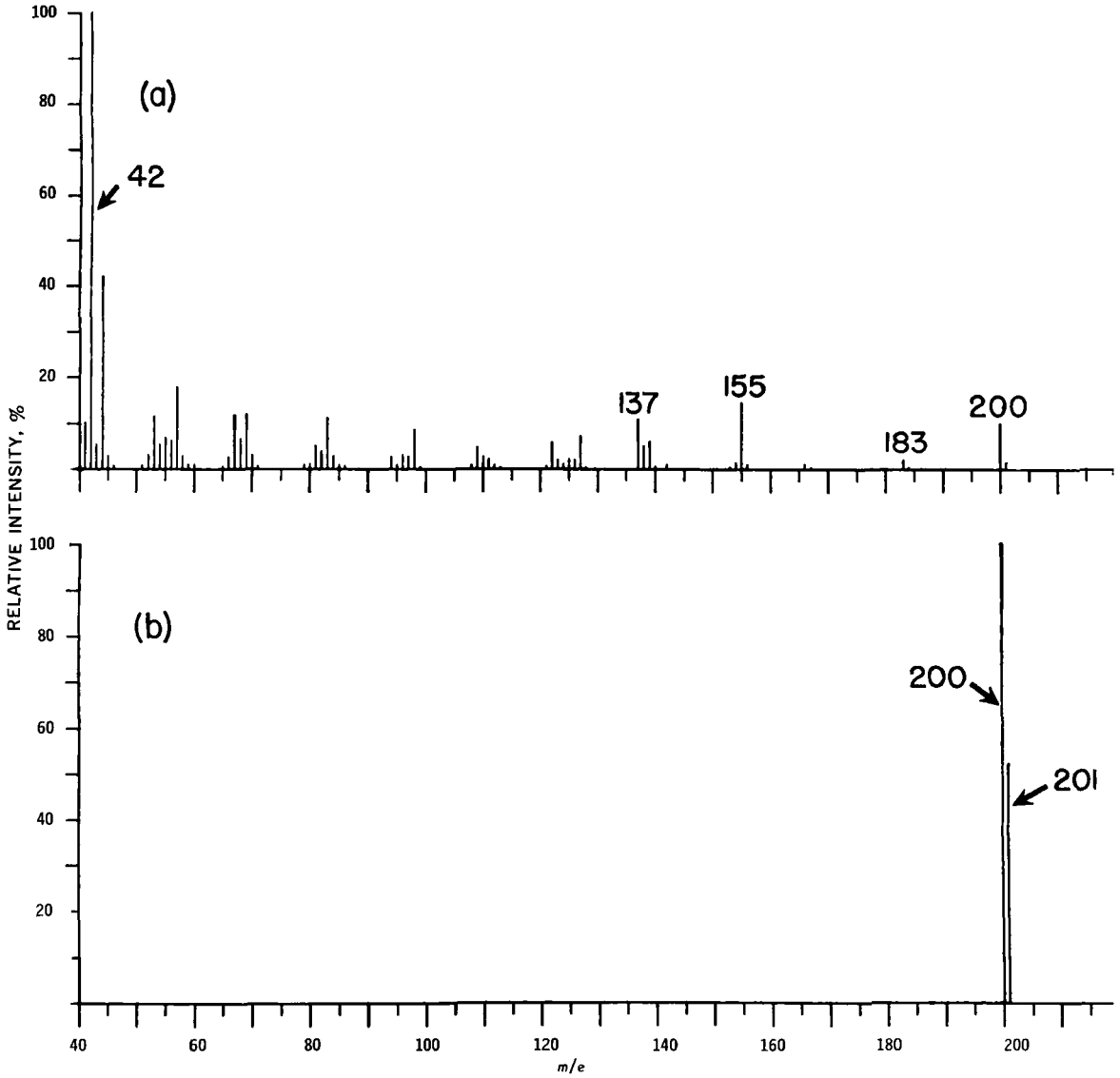


Figure 4—*Mass spectra of IV. (a) Electron impact, using a direct probe at a temperature of 240°. (b) Field desorption, using 26 mamp emitter heating current.*

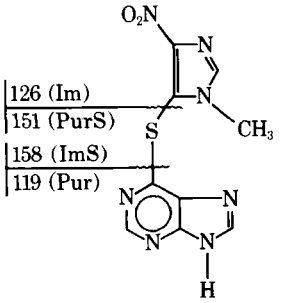
of NO from the molecular ion. The most intense high mass ion, *m/e* 231 ($C_9H_7N_6S$), is formed by the loss of NO_2. Fission of the sulfur–imidazole bond with rearrangement of a hydrogen to the purine moiety, $(PurS + H)^{\ddagger}$, results in an ion isomeric with 6-mercaptopurine at *m/e* 152 ($C_5H_4N_4S$). Cleavage between sulfur and the purine ring with retention of charge on the purine ring, $(Pur)^+$, accounts for the formation of the ion at *m/e* 119 ($C_5H_3N_4$). The base peak of the spectrum is *m/e* 42 (C_2H_4N).

In contrast, the field desorption mass spectrum gives an $(M + 1)^+$ ion [*m/e* 278 ($C_9H_8N_7O_2S$)] at T* (Fig. 1*b*). The T* is the optimal emitter heating current needed to obtain the most intense $M^{\ddagger}$ or $(M + 1)^+$ peaks. When the emitter current is raised (Fig. 1*c*), fragmentation is induced. The field desorption mass spectrum of I at 25 mamp emitter current is: *m/e* 278, 79%, $(M + 1)^+$; *m/e* 277, 22%, $M^{\ddagger}$; *m/e* 262, 24%, $M^{\ddagger} - CH_3$; *m/e* 261, 57%, $M^{\ddagger} - CH_4$; *m/e* 231, 100%, $M^{\ddagger} - NO_2$; *m/e* 152, 51%, $(PurS + H)^{\ddagger}$; *m/e* 130.5, 16%, doubly charged mass 261; *m/e* 131, 13%, doubly charged mass 262; *m/e* 119, 11%, $(Pur)^+$; and *m/e* 115.5, 22%, doubly charged mass 231.

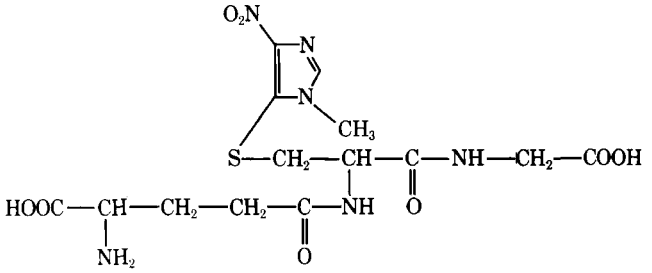
The fragments at *m/e* 231, 152, and 119 are common to both the electron impact and field desorption spectra of azathioprine. While both techniques offer fragments that can aid in characterizing I, the quasimolecular ion is only available in the field desorption spectra.

The electron impact spectrum of II (Fig. 2*a*) results primarily from pyrolytic decomposition. This spectrum is remarkably similar to the low-resolution field desorption spectrum taken at 27 mamp emitter heating current (Fig. 2*c*), with the exception of the molecular ion. The T* for II is 24 mamp (Fig. 2*b*). Under this mild thermal condition, only the $(M + 1)^+$ signal is seen. As the emitter temperature is increased, fragments are generated (17), yielding valuable structural information (Fig. 2*c*). The $(M + 1)^+$ peak is still large (26% relative intensity), and the base peak is now *m/e* 159 [$C_4H_5N_3O_2S$, $(ImS + H)^{\ddagger}$]. Also, $(ImS)^+$, *m/e* 158, and $(ImS + 2H)^+$, *m/e* 160, are present. The high-resolution field desorption mass spectrum has an ion at *m/e* 274 ($C_{10}H_{16}N_3O_6$), which is the loss of (ImS) from the molecule. Thus, the $(M + 1)^+$ and *m/e* 159 and 274 ions characterize this molecule.

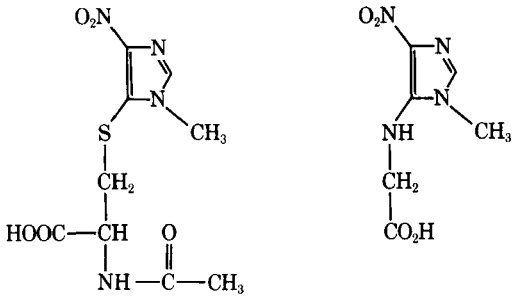
Compound III has a small molecular ion at *m/e* 288 (0.8% relative intensity) with electron impact mass spectrometry (Fig. 3*a*). The ion at *m/e* 209 has an elemental composition of $C_8H_9N_4OS$. Loss of ketene from *m/e* 209 yields an ion at *m/e* 167 ($C_6H_7N_4S$). The relative intensity of the $(ImS + H)^{\ddagger}$ ion is 7%. Loss of NO from the *m/e* 159 ion accounts for a small portion of the *m/e* 129 peak ($C_4H_5N_2OS$). The major portion of the *m/e* 129 peak is accounted for by the $(M^{\ddagger} - ImSH)$ ($C_5H_7NO_3$). The loss of H_2O from the *m/e* 129 ion gives *m/e* 111 ($C_5H_5NO_2$), and the loss of ketene yields an ion at *m/e* 87 ($C_3H_5NO_2$). The base peak of the electron impact mass spectrum is *m/e* 43 ($CH_3—C{\equiv}O^+$). Although the electron impact spectrum offers considerable structural information, the intensities of the high mass ions are small. The field desorption mass spectra of III have intense molecular ions (Figs. 3*b*



I ($C_9H_7N_7O_2S$, mol. wt. 277)



II ($C_{14}H_{20}N_6O_8S$, mol. wt. 432)



III ($C_9H_{12}N_4O_5S$, mol. wt. 288)

IV ($C_6H_8N_4O_4$, mol. wt. 200)

and 3*c*). At 24 mamp emitter current, $(M + 1)^+$, $(ImS)^+$, $(ImS + H)^+$, and $(ImS + 2H)^+$ appear. On the photoplate run, an ion was recorded at *m/e* 577 $(2M + 1)^+$, a common observation of cluster formation with field ionization.

Compound IV is relatively volatile, yielding a complete electron impact spectrum (Fig. 4*a*) with a substantial M‡ at *m/e* 200 (10%). The M‡ − CO_2H peak, *m/e* 155, is 15% as intense as the base peak, *m/e* 42 (C_2H_4N). An (M‡ − OH) ion at *m/e* 183 has a relative intensity of 2%. The ion at *m/e* 137 (11%) has the elemental composition $C_5H_5N_4O$. The field desorption spectrum of IV gave only M‡ and $(M + 1)^+$ ions (Fig. 4*b*). One explanation for the lack of induced fragmentation is that IV volatilizes from the emitter before enough thermal energy is transferred from the emitter to cause fragmentation.

To explore the potential of the field desorption method in its usefulness for the detection of drug metabolites, a sample of III isolated from the urine of rats given an oral dose of azathioprine was examined by field desorption mass spectrometry. Compound III was isolated by column chromatography[8] and purified by paper chromatography (3). Low-resolution field desorption mass spectrometry revealed the molecular weight of this metabolite. In an interval of the emitter heating current between 0 and 24 mamp, only two intense signals were obtained at *m/e* 288 (100% relative intensity) and 289 (40% relative intensity).

After this field desorption measurement was completed, observation of the emitter in a light microscope, 1:100 magnification, showed that the microneedles were still covered with a considerable amount of the sample. Consecutive high-resolution field desorption mass spectrometry with the double-focusing machine indicated that these residues are due to sodium and potassium salts, mainly phosphates, *e.g.*, *m/e* 80.974 $(HPO_3 + H)^+$, 96.969 $(HPO_4 + H)^+$, and 164.931 $(Na_3PO_4 + H)^+$. The photographically recorded spectrum showed Na^+ and $^{39,41}K^+$ peaks with saturated blackening.

In view of the importance of knowing the molecular weight of a metabolite, field desorption mass spectrometry is especially suited to studies of this type. The molecular weights of the compounds studied were easily accessible by field desorption mass spectrometry. In addition, valuable structural information can be obtained from fragments produced by higher emitter temperatures.

[8] The column used was DEAE Sephadex A-25 (acetate).

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