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METABOLISM OF THE AMINO ACID β-PYRAZOL-1-YLALANINE AND ITS PARENT BASE PYRAZOLE

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Abstract—β-Pyrazol-1-yl-DL-alanine, an uncommon amino acid from plants of the Cucurbitaceae, was fed to mice. Although pyrazole is known to affect the liver enzymes UDP-glucose dehydrogenase, UDP-glucuronyl transferase and UDP-glucuronic acid pyrophosphatase, and also depresses their liver glycogen concentrations, β-pyrazol-1-ylalanine had no such effects. β-Pyrazol-1-ylalanine could not be detected in the liver of the experimental animals but was present in the urine. No other change in urinary amino acid content was observed. Studies with [^{14}C]-β-pyrazol-1-yl-DL-alanine showed the administered amino acid was excreted over a 4-day period, 93% of the compound supplied was recovered. Similar recoveries were obtained with the L-enantiomer from cucumber seed. The metabolic inertness of β-pyrazol-1-ylalanine was also apparent in experiments involving subcutaneous injection of this compound.

Administration of pyrazole confirmed an earlier report of resultant increased activity of liver UDP-glucose dehydrogenase and UDP-glucuronyl transferase, and of the depression of activity of liver UDP-glucuronic acid pyrophosphatase. A concomitant 40% decrease in liver glycogen content was seen. The urine contained a novel metabolite, identified as a peptide conjugate of a pyrazole derivative. Mass spectrometry and p.m.r. spectroscopy indicate that this derivative is 3,4,4-trimethyl-5-pyrazolone. The amino acid consitutents are aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine and leucine. The urine of mice receiving pyrazole contained less free glycine and alanine than controls. From the results, it is concluded that pyrazole is not a catabolite of dietary β-pyrazol-1-ylalanine but to the contrary, the amino acid is essentially excreted unchanged.

Formation of 3,4,4-trimethyl-5-pyrazolone from pyrazole would imply *C*-methylation, a process that has not been previously observed in a mammalian detoxication context.

β-Pyrazol-1-yl-L-alanine (**1**), an isomer of histidine was first isolated [1] from the juice of water melon (*Citrullus vulgaris*). Elucidation of its structure was described by Noe and Fowden [2, 3]. The compound has since been found, together with the peptide γ-L-glutamyl-β-pyrazol-1-yl-L-alanine in fruits, seeds and juice of many other species of Cucurbitaceae, including cucumber [e.g. ref. 4]. The free amino acid is present to the extent of 1 g/kg of water melon seeds [3]. Biosynthetically, it arises from pyrazole (**2**) and *O*-acetylserine [2, 4, 5]. Pyrazole and its derivatives are of special interest biochemically in that they are rare examples of naturally occurring structures containing N–N bonds. Whereas the biosynthetic origin of the pyrazole ring has been investigated [6], little is known of the metabolism of this compound and its derivatives in mammals. As a constituent of cucurbits however, β-pyrazol-1-ylalanine must be considered a dietary amino acid. Together with the discovery [7] of a microbial enzyme capable of degrading β-pyrazol-1-ylalanine to pyrazole, the foregoing observations and the reported effects of pyrazole on mammalian metabolism led to the present investigation. Its aim was elucidation of the metabolic fate of β-pyrazol-1-ylalanine and of its parent base, pyrazole.

Pyrazole is a potent competitive inhibitor of liver alcohol dehydrogenase [8–10] and also inhibits the microsomal ethanol-oxidizing system. In addition it inhibits catalase and the transport of reducing equivalents into the mitochondrion [11]. Chronic administration of the compound induces ultrastructural changes in rat liver [12] and affects brain noradrenalin concentrations [13]. Pyrazole affects enzymes, such as NADP-cytochrome *c* reductase, involved in hydroxylation processes [14] and also those enzymes involved in glucuronidation. The activities of UDP-glucose dehydrogenase, UDP-glucuronosyl transferase, and L-gulonate dehydrogenase, are markedly enhanced, whereas the activities of UDP-glucuronic acid pyrophosphatase,

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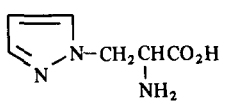

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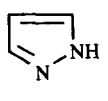

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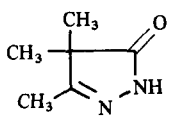

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β-glucuronidase, and D-glucuronolactone dehydrogenase are decreased [14].

Although nothing is known of the mammalian metabolism of pyrazol-1-ylalanine and of pyrazole, the metabolic fate of the pyrazolinone drug antipyrine has been investigated. Antipyrine (2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) is mainly oxidized to 4-hydroxyantipyrine [15–19] but about 5% is eliminated unchanged [17] and another fraction is oxidized, at the 3-methyl group, to give 3-hydroxymethyl-2-methyl-1-phenyl-3-pyrazolin-5-one [18, 19].

MATERIALS AND METHODS

Analytical grade chemicals and solvents were purchased from BDH Chemicals Ltd. (Poole, Dorset). Pyrazole, amino acids, NAD^+ (Na salt), UDPG (Na salt), UDP-glucuronic acid, D-amino acid oxidase, catalase, and glycogen were obtained from Sigma (London) Chemical Co. Ltd. (Poole, Dorset). L-[U-^{14}C]Serine was purchased from Amersham International plc (Amersham, Bucks). Seeds of *Cucumis sativus* cv. Ridge perfection were obtained from Asmer Seeds Ltd. (Leicester).

Preparation and isolation of β-pyrazol-1-yl-DL-alanine. β-Pyrazol-1-yl-DL-alanine was prepared by means of the pyridoxal model enzyme system described by Dunnill and Fowden [4]. Pyrazole (1.47 mmol), serine (0.19 mmol), pyridoxal 5-phosphate (37 μmol) and $Al_2(SO_4)_3$ (7.9 μmol) were dissolved in a final volume of 3 ml of 0.1 M K acetate buffer (pH 4.7) and heated at 60° for 20 hr. β-Pyrazol-1-ylalanine was purified by cation-exchange chromatography on a column (60 × 1.6 cm dia.) of Dowex-50 (W) X8; H^+; 200–400 mesh. The elution procedure was based on that of Moore and Stein [20]. After equilibration of the column with 0.2 M Na citrate buffer (pH 2) the reaction mixture was adjusted to pH 2 with acetic acid and loaded at 30 ml/hr. Elution was with the same citrate buffer at a flow rate of 25 ml/hr. Fractions (10 ml) were collected automatically. Fractions 9–36 were pooled, evaporated *in vacuo* to dryness and redissolved in 20 ml water. To remove traces of pyridoxal phosphate and of the citrate buffer, the solution was passed through a similar size column of Dowex-50 (W) X8; NH_4^+; 200–400 mesh. After washing the column with 4 bed volumes of water, the β-pyrazol-1-ylalanine was eluted in 0.25 M ammonia solution. The purity of the product was checked by paper and thin-layer chromatography, and high voltage paper electrophoresis. [^{14}C]-Pyrazol-1-ylalanine, labelled uniformly in the alanine moiety, was prepared from pyrazole and L-[U-^{14}C]serine by essentially the same procedure as that described above except that 0.06 μCi of L-[U-^{14}C]serine was added to the non-radioactive serine (0.19 mmol) in the reaction mixture.

Chromatography and electrophoresis. Solvent systems used for paper and thin-layer chromatography were: (1) butan-1-ol saturated with 3 M ammonia solution, (2) butan-1-ol/acetic acid/water (90:10:29, by vol), (3) butan-1-ol/acetic acid/water (12:3:5, by vol), (4) propan-2-ol/ammonia (sp. gr. 0.88)/water (7:1:2, by vol), (5) phenol/water (160 g/40 ml), (6) butan-1-ol/acetone/diethylamine/water (70:70:14:35, by vol), (7) butan-1-ol/acetone/ammonia (sp. gr. 0.88)/water (10:10:5:2, by vol), (8) propan-2-ol/formic acid/water (20:1:5, by vol). For routine detection of β-pyrazol-1-ylalanine, two-dimensional chromatograms were run with solvent 1 in the first dimension and solvent 2 in the second. The R_f values for β-pyrazol-1-ylalanine in solvents 1, 2 and 5 were 0.08, 0.22 and 0.78, respectively. During high voltage electrophoresis, with a current of 50 mA and using 3 MM Whatman paper, β-pyrazol-1-ylalanine migrated 6 cm/hr towards the cathode at pH 2. High voltage paper electrophoresis was on Whatman 3 MM paper using either a Na borate buffer (0.05 M, pH 10) or a formic acid/acetic acid buffer (pH 2). The latter was 0.8 M with respect to formic acid and 0.7 M with respect to acetic acid.

Amino acids and peptides were located using 0.2% (w/v) ninhydrin in acetone. Ehrlich's reagent was prepared by dissolving 1 g *p*-dimethylaminobenzaldehyde in 100 ml of acetone/HCl (9:1, v/v). Pauly reagent was prepared from diazotized sulphanilic acid [21].

Detection and measurement of radioactivity. Radioactive areas were located on chromatograms and electrophoretograms using the 'spark-chamber' apparatus (Birchover Instruments, Letchworth). For measurement of radioactivity aqueous samples (0.5 ml) were added to 5 ml aliquots of RiaLuma scintillation fluid (LKB Ltd., London). Duplicate samples were counted in a Model 1217 Rack-Beta liquid scintillation spectrometer (LKB-Wallac Ltd., London). Each sample was counted for 10 min. The limit of detection of ^{14}C-labelled urinary metabolites was 150 dpm. Quench correction was by the external standard channel ratio method.

Determination of metabolite concentrations. Amino acid concentrations were determined colorimetrically by the quantitative ninhydrin method of Lee and Takahashi [22]. Pyrazole concentrations were determined by a colorimetric procedure [23] which uses the yellow colour produced by pyrazole in the presence of trisodium pentacyanoaminoferrate.

The procedure for determining the concentration of the D-enantiomer of β-pyrazol-1-ylalanine made use of the stereospecificity of D-amino acid oxidase (EC 1.4.3.3). The enzyme, which is specific for the D-enantiomer [24] was incubated with the substrate at 37° for 1–4 hr. The incubation mixture contained, in addition to the enzyme, catalase (1 unit) and 0.2 M Tris buffer (pH 8.3, oxygen-saturated).

Liver glycogen concentrations were determined by the anthrone method of Plummer [25].

Animal studies. Male mice of the 'To' strain were used throughout. Animals weighing between 30 and 40 g were kept, in groups of three, in metabolic cages. For oral administration of compounds under investigation, animals were fasted overnight and then supplied with food containing the compound. For β-pyrazol-1-ylalanine studies, doses of 200 mg/kg body weight were fed daily for 4–8 days. With cucumber seed, the finely milled seed was mixed with equal amounts of milled food. For subcutaneous injection, pyrazole or β-pyrazol-1-ylalanine was dissolved in normal saline and administered at a dosage of

100 mg/kg body weight daily. Control animals were injected with corresponding volumes of saline (1.15% w/v KCl).

Measurement of enzymic activities. The activity of UDP-glucuronic acid pyrophosphatase (EC 3.6.1.9) was measured in the way described by Marselos *et al.* [14]. UDP-glucuronyl transferase (EC 2.4.1.17) was assayed by the procedure of Isselbacher *et al.* [26] and UDP-glucose dehydrogenase (EC. 1.1.1.22) by the method of Goldberg *et al.* [27]. The activity of histidine-ammonia lyase (EC. 4.3.1.3) was measured by the method of Tabor and Mehler [28].

Desalting of urine samples. Urine samples were desalted by ion-exchange chromatography. A column (5 cm × 1 cm diam.) of Dowex-50 × 8; H^+; 200–400 mesh, was prepared. The urine sample (1 ml) was loaded onto the column and washed through with 15 ml of 2 M ammonia and the eluate collected. After evaporation *in vacuo* to dryness, the residue was redissolved in 1 ml water and taken for chromatographic analysis.

Hydrolysis of peptides. Peptides were hydrolysed for 72 hr at 120° in sealed glass ampoules, each containing a 200–500-fold excess of 6 M HCl.

NMR and mass spectra. NMR spectra were obtained with a Varian XLFT-100 instrument with a reference frequency of 100.08 MHz. Microgram quantities of the sample were dissolved in minimal volumes of $CDCl_3$ and spectra calibrated with tetramethylsilane as internal standard. The field was scanned at ambient temperature: 676 scans. Mass spectra were recorded using an AEI MS-9 mass spectrometer.

RESULTS AND DISCUSSION

*Metabolism of β-pyrazol-1-*DL*-ylalanine*

Groups of twelve mice (3/metabolic cage) were fasted overnight before administration of the amino acid began. A dosage of 200 mg of β-pyrazol-1-DL-ylalanine/kg body weight was fed daily, mixed with a small amount of pulverized food. In one series of experiments, feeding was continued for a total of 4 days and in a second series, for a total of 8 days. Control animals were treated in the same way except that β-pyrazol-1-ylalanine was omitted from the diet. Addition of β-pyrazol-1-ylalanine in no way deterred the animals from consuming all the food offered.

Examination of tissue samples (Table 1) taken after 4 days and after 8 days, showed no significant effect by the administered compound on those enzymes of the glucuronic acid pathway known to be affected by pyrazole [14]. These included UDP-glucose dehydrogenase, UDP-glucuronyltransferase and UDP-glucuronic acid pyrophosphatase. Daily administration of β-pyrazol-1-ylalanine by subcutaneous injection, for 4 or 8 days at a dosage of 100 mg/kg body weight, also failed to show any significant effect on the activity of these enzymes (Table 1). It was concluded that pyrazole is not likely to be a major catabolite of β-pyrazol-1-ylalanine. This conclusion was supported by the observation that β-pyrazol-1-ylalanine administration did not significantly decrease the liver glycogen concentration as pyrazole would have been expected to do [14].

Chromatographic analysis of liver tissue from animals receiving β-pyrazol-1-ylalanine showed no detectable amount of this amino acid. The total urine output from the animals was collected and pooled over a 4-day period and over an 8-day period. After de-salting, samples were analysed for amino acid content. The results showed that β-pyrazol-1-ylalanine was present in both the 4- and 8-day urine samples from the experimental animals and absent from that of the corresponding controls. Apart from this, there was no apparent qualitative or quantitative difference between any of the urine samples. Histidine, serine, alanine and minor amounts of other amino acids were present in all the samples examined. Since β-pyrazol-1-ylalanine is an isomer of histidine, it was of interest that there was no obvious disturbance in histidine metabolism in the experimental animals. *In vitro* experiments with β-pyrazol-1-DL-ylalanine showed that it had no detectable effect on the enzyme histidine-ammonia lyase.

Since the β-pyrazol-1-ylalanine supplied to the animals was a synthetic racemic mixture, it was not clear from the foregoing observations whether the L-enantiomer was being metabolized and the D-enantiomer excreted or whether the amino acid in the urine was the racemate. A sample of the excreted amino acid was therefore isolated, quantitatively determined and incubated with stereospecific D-

Table 1. Examination of hepatic enzymes of the glucuronic acid pathway for effects of β-pyrazol-1-ylalanine (β-PA) administration

Enzyme	Mode of β-PA administration	Activity (pkat/g liver) Controls	Treated animals
UDP-glucose dehydrogenase	Diet	33.7 ± 2.2	35.4 ± 3.1
(E.C. 1.1.1.22)	Injection	35.6 ± 2.5	37.0 ± 3.3
UDP-glucuronyl transferase	Diet	1.02 ± 0.1	1.10 ± 0.1
(E.C. 2.4.1.17)	Injection	1.03 ± 0.1	1.02 ± 0.1
		(nkat/g liver)	
UDP-glucuronic acid pyrophosphatase	Diet	4.23 ± 0.3	4.30 ± 0.4
(E.C. 3.6.1.9)	Injection	4.19 ± 0.4	4.18 ± 0.4

β-Pyrazol-1-ylalanine was administered for 8 days either by diet (200 mg/kg body weight/day) or by subcutaneous injection (100 mg/kg body wt/day). Data are means ± S.D. for replicate sets, each of three mice.

Table 2. Recovery from urine of [^{14}C]β-pyrazol-1-yl-DL-alanine supplied in the diet to mice

Day	Total radioactivity in β-pyrazol-1-ylalanine isolated (dpm × 10^{-3})	Recovery (%)
1	51.61	74
2	8.42	12
3	2.80	4
4	1.77	3
1–4 (total)	64.60	93

[U-^{14}C-alanine]β-pyrazol-1-yl-DL-alanine (69.07×10^3 dpm; specific activity 413 dpm/μmole) was fed to mice on day 0. The total urine output was collected every 24 hr. The figures shown, which represent a typical set of data for three mice, were reproducible.

amino acid oxidase. In a pilot experiment, the procedure (see Experimental) gave 46% oxidation of an authentic DL-mixture of serine under the same conditions. With the β-pyrazol-1-ylalanine sample from the urine, results indicated 55% oxidation. Allowing for experimental error, this shows that the excreted amino acid is the racemate originally administered. Comparison of the amount of β-pyrazol-1-ylalanine in the total urine collection with the amount originally supplied showed that 90% had been recovered unchanged. To assess whether this meant that none of the β-pyrazol-1-ylalanine had been metabolized, the compound was synthesized from [U-^{14}C]alanine and the radioactive amino acid used to repeat the feeding experiment. The results (Table 2) showed that 74% of the compound presented to the animals was excreted on the first day of ingestion and that by the end of the 4-day period, 93% of the original amount had been recovered in the urine.

In another feeding experiment, animals (3) were fed crude β-pyrazol-1-yl-L-alanine in the form of finely milled cucumber seed (38 g seed) mixed with pulverized, standard mouse diet. Quantitative analysis showed that the 38 g of seed contained 1.5 mmole β-pyrazol-1-yl-L-alanine. This modified diet was supplied daily for a total of 8 days and the total urine output over the period was collected and pooled. Again there was virtually a quantitative recovery of the amino acid, confirming that the L-enantiomer is not metabolized and that the results of the earlier feeding experiments with the racemate were not attributable to inhibition of the metabolism of the L-compound by its D-isomer.

As a cross-check on the apparent metabolic inertness in this mammalian system of β-pyrazol-1-ylalanine, the compound was administered subcutaneously to mice at a dosage of 100 mg/kg daily for 4 days. Urine was collected every 24 hr, desalted and chromatographed as before. As in the feeding experiments, the only detectable difference between the urine of control and injected animals was the presence in the urine from the latter of β-pyrazol-1-ylalanine. Examination of the liver enzymes, UDP-glucose dehydrogenase, UDP-glucuronyl transferase and UDP-glucuronic acid pyrophosphatase showed that as in the feeding experiments, these pyrazole-sensitive enzymes had not been affected. No pyrazole could be detected in the urine.

Metabolism of pyrazole

There is evidence that in the plants which produce β-pyrazol-1-ylalanine, synthesis of this amino acid serves as a detoxication mechanism for pyrazole [29]. As discussed above, the latter compound has pronounced physiological effects on mammalian tissues and it would therefore seem probable that these tissues also have a pyrazole detoxication mechanism. To investigate this, and to shed light on pyrazole catabolism, a similar series of experiments to those described above were set up and pyrazole was administered subcutaneously for 4 days at a daily dosage of 100 mg/kg body weight. As before, the activities of the liver enzymes UDP-glucose dehydrogenase, UDP-glucuronyl transferase and UDP-glucuronic acid pyrophosphatase were assayed *in vitro* at the end of the 4-day period. The results (Table 3) confirm previous reports [14] of an increase in the activity of UDP-glucose dehydrogenase (178% of control) and UDP-glucuronyl transferase (150% of control) and a decrease in the activity of UDP-glucuronic acid pyrophosphatase (53% of control). Also as previously observed by Marselos *et al.* [14], the liver glycogen concentration was found to have substantially decreased following pyrazole treatment. In a typical experiment in the present series, there was a decrease from 159 mg/g of tissue to 96 mg/g (i.e. a 40% decrease).

To see if the animal tissue possessed a pyrazole-detoxication mechanism in any way comparable to the plant system, i.e. involving conjugation with an amino acid, urine from the pyrazole-treated animals was collected, desalted, and analysed for amino acid content. Comparison by 2-dimensional thin-layer chromatography (cellulose layers, solvent 7 followed by solvent 8) of urine samples from animals receiving

Table 3. Activity of enzymes of the D-glucuronic acid pathway in mouse liver after administration of pyrazole

Enzyme	Control mice (pkat/g liver)	Pyrazole mice (pkat/g liver)
UDP-Glucose dehydrogenase (E.C. 1.1.1.22)	33.0 ± 2.1	58.8 ± 1.8
UDP-Glucuronyl transferase (E.C. 2.4.1.17)	1.0 ± 0.1	1.5 ± 0.1
UDP-Glucuronic acid pyrophosphatase (E.C. 3.6.1.9)	4.3 ± 0.3	2.3 ± 0.4

Pyrazole was injected subcutaneously for 4 days at a dosage of 100 mg/kg body wt/day. At the end of this time, liver tissue was removed for the enzymic assays. Figures shown are means ± S.D. for replicate sets, each of three animals.

Table 4. Comparison of published n.m.r. spectral data for 3,4,4-trimethyl-5-pyrazolone with that for the putative biological sample

Sample	Solvent	α(ppm)	Assignment	Reference
Synthetic	$CDCl_3$	1.12s	gem-dimethyl	Jones *et al.* (1963)
		1.93s	vinylic methyl	
Synthetic	$CDCl_3$	1.10s	gem-dimethyl	Evans *et al.* (1965)
		1.98s	vinylic methyl	
Biological	$CDCl_3$	1.14s	gem-dimethyl	
		2.00s	vinylic methyl	

The 'biological' sample was isolated from the hydrolysate of the peptide metabolite of pyrazole. With all three samples, the internal standard was TMS.

pyrazole, with samples from control animals, showed no detectable trace of β-pyrazol-1-ylalanine. However, there was very clearly a major new metabolite in the urine from the experimental animals. On paper and thin-layer plates this gave a purple reaction with 0.2% (w/v) ninhydrin in acetone. The reaction took place within a few minutes without heating which is indicative of presence of a 2-amino acid. The compound could also be detected on chromatograms by its absorption of u.v.-light.

In aqueous solution (pH 2), the u.v. absorption spectrum of the metabolite exhibits λ_{max} 273_{nm}, pH 10). Chromatography on Whatman No. 1 paper in solvent 1 gave an R_f of 0.02, in solvent 2 its R_f was 0.2, in solvent 3, R_f 0.27, in solvent 4, R_f 0.28, and in solvent 5, R_f 0.48. With the Pauly reagent [21] the unknown gave a red-brown colour, and with Ehrlich's reagent [21], a yellow colour resulted. During high voltage electrophoresis on paper (Whatman 3 MM; 2 kV; 50 mA; 1 hr) in a formic acid/acetic acid buffer at pH 2, the compound migrated 7.2 cm towards the cathode. In a sodium borate buffer (pH 10) but otherwise similar conditions, the sample migrated 5.5 cm towards the anode.

In addition to the new metabolite in the urine of mice injected with pyrazole, there were quantitative differences apparent on the thin-layer plates between the experimental and control urine samples. Desalted samples were taken for analysis in an automatic amino acid analyser. The results showed a peak, eluting in the valine region, which although present in both urine samples, was significantly larger in the sample from the pyrazole-treated animals. It was also observed that the peaks of glycine and alanine were significantly smaller in the urine sample from the experimental animals. Further examination of the peak eluting in the valine region showed that it was not valine, neither was it any of the commonly occurring amino acids known to elute in this region of the elution sequence. These included tyrosine, tryptophan, histidine and phenylalanine. This compound was not identified.

Chromatographic and electrophoretic comparison of the new major pyrazole metabolite indicated that it was not one of the common amino acids. Its chromatographic and electrophoretic behaviour, however, indicated the possibility that it is a peptide. This was confirmed by acid–hydrolysis followed by thin-layer chromatography and electrophoresis of the hydrolysate. Examination of the hydrolysate in the amino acid analyser identified 9 amino acids, *viz.* aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, and leucine. Chromatography of the hydrolysate on paper in solvents 2, 3, 5 and 6 showed that in addition to the constituent amino acids, there was a ninhydrin-negative, u.v.-absorbing component R_f solvent 2, 0.08; R_f solvent 3, 0.16; R_f solvent 5, 0.06: R_f solvent 6, 0.07. The compound was purified by sequential chromatography as a band in solvents 2, 3, 5 and 6. In aqueous solution at pH 2, it exhibited $\lambda_{max} \simeq$ 212 nm. During high voltage electrophoresis at pH 2 under the conditions described in the Methods section, the compound migrated 9 cm/hr towards the cathode.

The mass spectrum of the unknown ninhydrin-negative component of the hydrolysate was recorded at 70 eV/250° using an AEI MS-9 mass spectrometer. The most prominent features of the spectrum were peaks at *m/z* 254, 128, 127, 112, 99, 97, 84, 70, 57, 53, 43, 41 and 27. With the exception of *m/z* 254, these data correspond closely with the characteristic fragmentation pattern of 3,4,4-trimethyl-5-pyrazolone (3) recorded by Desmarchelier and Johns [30]. The peak at *m/z* 254 $[M + H^+]_2$ is attributable to dimerization of the sample during isolation. Pyrazoles unsubstituted at the 1-position are known to exhibit particularly strong association to give dimers during preparation of crystalline samples [31]. Fragmentation involves initial loss of CH_3 giving *m/z* 112 (3,4-dimethyl-5-pyrazolone) and loss of C_2H_5 to yield a fragment ion *m/z* 97. Loss of CO from 3,4-dimethyl-5-pyrazolone gives rise to *m/z* 84 ($C_4H_8N_2$). There are three possible fragmentation pathways for this, (a) loss of CH_3CN (*m/z* 41) to yield ethyleneimine (C_2H_5N, *m/z* 43), (b) loss of CH_3NH_2 to give propene nitrile (C_3H_3N, *m/z* 53), and (c) loss of HCN resulting in formation of 2-methylaziridine (C_3H_7N, *m/z* 57). The latter loses CH_3 to give aziridine (C_2H_5N, *m/z* 43). Elimination of C_2H_5 from *m/z* 128 yields *m/z* 99 and loss of N_2H then gives 2-butenal (C_4H_6O, *m/z* 70). Elimination of ketene ($CH_2{=}C{=}O$) from *m/z* 99 gives 2-methylaziridine (*m/z* 57). As discussed by Desmarchelier and Johns [30] the mass spectrum of 3,4,4-trimethyl-5-pyrazolone is significantly different from that of its isomer 1,2,3-trimethyl-5-pyrazolone, in which structure there is *N*-methylation.

On the basis of the foregoing observations and considerations, the unknown hydrolytic product from the peptide was tentatively identified as 3,4,4-trimethyl-5-pyrazolone. Examination of the com-

pound by NMR gave a proton magnetic resonance spectrum (Table 4) compatible both with the tentative identification made by mass spectrometry and with previously published NMR data for 3,4,4-trimethyl-5-pyrazolone [32, 33]. The spectrum showed a signal at 1.14 δ and another at 2.00 δ attributable, respectively, to a gem-dimethyl and a vinylic methyl. The relative number of protons associated with these signals was recorded as 2:1 (gem-dimethyl:methyl). From the amino acid analysis, mass spectrum and p.m.r. data, it is therefore concluded that the main pyrazole-excretory product of mice subcutaneously injected with pyrazole is a peptide conjugate containing a pyrazole derivative, with the proposed structure 3,4,4-trimethyl-5-pyrazolone and the amino acids aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, and leucine.

Although *C*-methylation, e.g. synthesis of thymidylate and 5-methylcytidylate, is known to occur in mammalian tissues, conversion of pyrazole to 3,4,4-trimethyl-5-pyrazolone as part of a mammalian detoxication mechanism is of interest since little is known of *C*-methylation in this context. Published information on methylation in detoxication is currently confined to *N*- and *O*-methylation reactions catalysed by S-adenosyl-L-methionine methyl transferases [34]. Formation of a methylated pyrazolone raises questions as to whether the hydroxylation of pyrazole precedes or follows methylation, which *C* is methylated first, C_3 or C_4, and which methyl donor is involved. There is also the problem of whether methylation occurs before or after conjugation with the peptide.

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