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Mercapturic Acid Formation During the Metabolism of Arecoline and Arecaidine in the Rat

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1. In the rat, arecoline is converted into arecaidine and both compounds are converted into *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine. 2. The structure of the metabolite was established by (*a*) synthesis, (*b*) conversion into *N*-acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine methyl ester, which was chromatographically identical with the synthetic material, and (*c*) n.m.r.- and i.r.-spectral analysis of the 3-methoxycarbonyl derivative. 3. In ethanolic solution, or in phosphate buffer at pH 7·0, arecoline reacted with *N*-acetyl-L-cysteine to give *N*-acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine; under similar conditions, arecaidine reacted more slowly to give *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine. 4. The reaction between arecoline and glutathione or *N*-acetyl-L-cysteine occurred maximally at neutral pH and decreased rapidly with increasing acidity. At neutral pH, the reactions were bimolecular and second-order when the reactants were in approximately equimolar concentrations and pseudo-unimolecular first-order when arecoline was in large excess. 5. Consideration of the pK_a values and degrees of ionization of the reactants and the effect of pH on the stoicheiometry of reaction between arecoline and glutathione or *N*-acetyl-L-cysteine indicated that reaction between un-ionized species occurred more readily than nucleophilic addition (Ad_N) reactions involving charged intermediates.

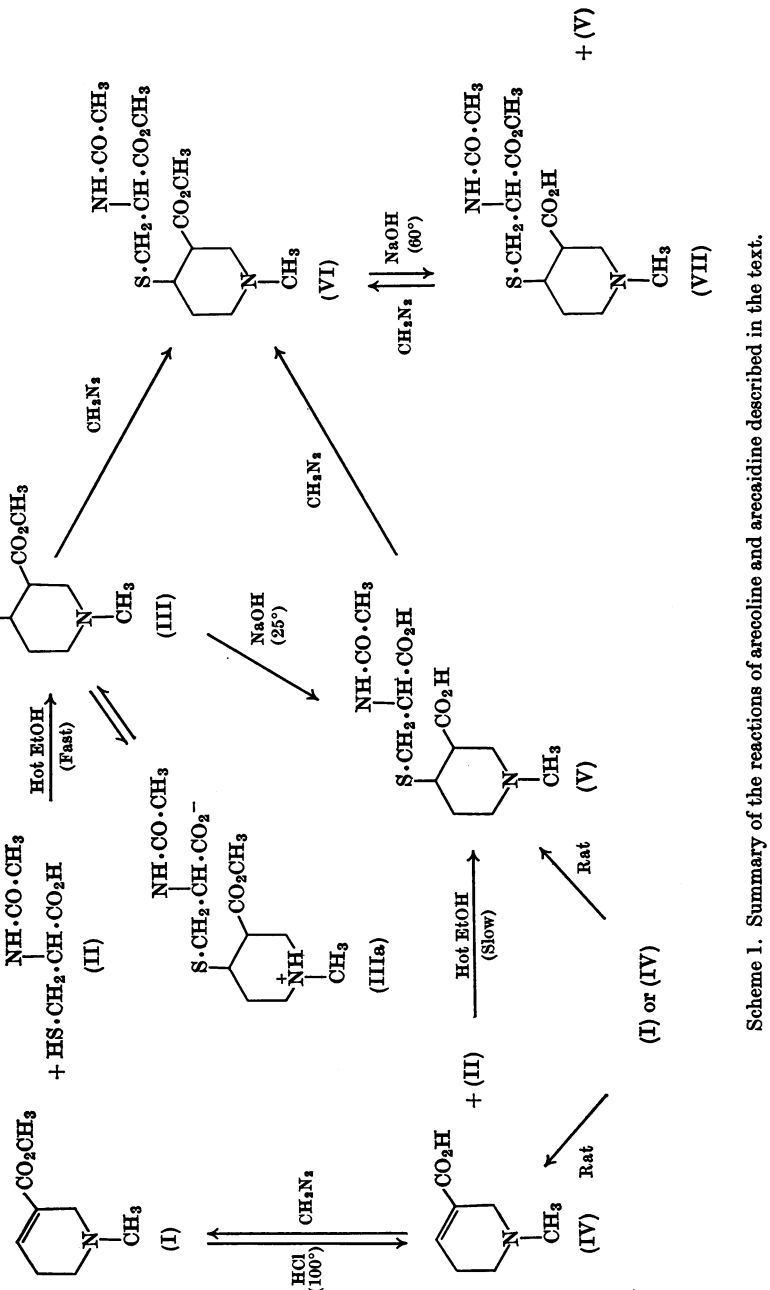
Arecoline (1,2,5,6-tetrahydro-1-methylnicotinic acid methyl ester; I) (see Scheme 1) is one of the alkaloids of betel nuts (*Areca catechu* L.) that has been used in veterinary medicine as a cathartic and taeniacide. The chewing of betel-nut preparations is probably a cause of cancer of the mouth. Nieschulz & Schmersahl (1968) showed that the lime that is a component of the betel mixtures chewed extensively in the Orient hydrolyses arecoline into the corresponding free acid (arecaidine; IV). The acid retains the stimulating properties but not the typical parasympathomimetic properties of the parent ester. Arecoline is also hydrolysed into arecaidine by rat liver homogenates (Nieschulz & Schmersahl, 1968). The present paper shows that rats metabolize arecoline into arecaidine and convert both substances into a mercapturic acid, i.e. into *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine (V).

MATERIALS AND METHODS

Spectra and optical rotation. The i.r. spectra were determined on a Perkin–Elmer 521 grating spectrophotometer, u.v. spectra on a Unicam SP. 800 u.v. spectrometer, n.m.r. spectra on a Perkin–Elmer R10 n.m.r. spectrometer and optical rotation on a Perkin–Elmer 141 polarimeter.

Animals. Adult Chester Beatty rats (mean body wt. 200g.) were kept in metabolism cages designed for the separate collection of urine and faeces. Urine was collected daily under toluene in receivers immersed in solid CO_2–acetone and stored at −5°. Arecoline hydrochloride was administered as an aq. 2% (w/v) solution by intraperitoneal injection, or as a 0·2% (w/v) solution in the drinking water; arecaidine hydrochloride was administered as an aq. 2·5% (w/v) solution by intraperitoneal injection, or as a 0·5% (w/v) solution in the drinking water. Food (Diet 86; Plowco Feeds Ltd., South Godstone, Surrey) and water were available *ad lib.*

Chromatography. Whatman no. 1 chromatography paper (unless otherwise stated) was employed for overnight downward development in the following solvent systems: (*a*) butan-1-ol–propan-1-ol–aq. 2M-NH_3 (2:1:1, by vol.); (*b*) butan-1-ol–acetic acid–water (12:3:5, by vol.); (*c*) butan-1-ol–acetic acid–water (2:1:1, by vol.). For t.l.c., glass plates were coated with films of silica gel G (E. Merck A.-G., Darmstadt, W. Germany) of 0·25mm. thickness and the chromatograms were developed in (*d*): ethanol–aq. M-NH_3–light petroleum (b.p. 40–60°) (6:1:1, by vol.). For the detection of compounds on chromatograms, the following reagents were used: (i) ammoniacal aq. 2% (w/v) $AgNO_3$; (ii) 0·1M-$K_2Cr_2O_7$–acetic acid (1:1, v/v) followed by 0·1M-$AgNO_3$ (Knight & Young, 1958); (iii) 0·05% (w/v)



Scheme 1. Summary of the reactions of arecoline and arecaidine described in the text.

Table 1. *R_F values of reference compounds*

For details of preparations etc. see the text.

	Compound	Solvent ...	R_F (a)	(b)	(c)	(d)
(I)	Arecoline		0·85	0·67	0·74	0·61
(III)	*N*-Acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine		0·27	0·49	0·65	0·54
(IV)	Arecaidine		0·08	0·40	0·64	0·20
(V)	*N*-Acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine		0·01	0·33	0·46	0·23
(VI)	*N*-Acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine methyl ester		0·77	0·64	0·70	0·81
(VII)	*N*-Acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine methyl ester (?)		0·09	0·44	0·61	—

ninhydrin in acetone followed by heating the chromatograms at 85–90° for 2–10min.; (iv) aq. 5% (w/v) $KBiI_4$–10M-HCl (200:1, v/v) (Bartley, 1954). A Chromatolite lamp (Hanovia Ltd.) was used as a source of u.v. light.

Sulphides and thiols (II, III and V–VII; see Scheme 1) gave yellow to brown spots with reagent (ii); *N*-methylpiperidines (I and III–VII) gave faint-pink to red spots with reagent (iv); arecoline (I) and arecaidine (IV) reduced reagent (i) after 3–4hr.

Chemicals. Arecoline was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and *N*-acetyl-L-cysteine from British Drug Houses Ltd., Poole, Dorset.

Arecaidine (IV). Arecoline (10g.) in 6M-HCl (250ml.) was heated in a flask fitted with a water-cooled reflux condenser at 100° for 8hr. The mixture was evaporated to dryness *in vacuo* and the residue recrystallized to constant m.p. from aq. 80% (v/v) ethanol to yield colourless plates of arecaidine hydrochloride (10·8g., 95%), m.p. 265° (decomp.) (Found: C, 47·7; H, 7·1; Cl, 19·8; N, 8·05. Calc. for $C_7H_{12}ClNO_2$: C, 47·3; H, 6·8; Cl, 20·0; N, 7·9%). Wohl & Johnson (1907) report m.p. 262° (decomp.), on rapid heating, for this compound. A mixture of silver acetate (1·7g.) in water (150ml.) at 60° was treated with a solution of arecaidine hydrochloride (1·9g.) in water (150ml.) at 60° and filtered. The filtrate was treated with H_2S and filtered, the filtrate evaporated *in vacuo* and the residue recrystallized from aq. 80% ethanol to give arecaidine monohydrate (1·0g., 61%) as colourless needles, m.p. 222° (decomp.). A solution of arecaidine hydrochloride (1·8g.) and NaOH (0·4g.) in water (10ml.) was evaporated *in vacuo* and the residue recrystallized from aq. 80% ethanol to yield arecaidine monohydrate (1·3g., 80%), m.p. 222° (decomp.). After drying at 100°/15mm. Hg over P_2O_5 for 3hr., this gave arecaidine, m.p. 231° (decomp.) (Found: C, 59·4; H, 8·1; N, 9·6. Calc. for $C_7H_{11}NO_2$: C, 59·55; H, 7·85; N, 9·9%). Wohl & Johnson (1907) report m.p. 222° (decomp.) and 232° (decomp.) for the monohydrate and anhydrous compounds respectively.

N-Acetyl-S-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine (III). A solution of arecoline (1·6g.) and *N*-acetyl-L-cysteine (1·7g.) in ethanol (100ml.) was heated under reflux for 6hr. and evaporated *in vacuo*. The residue was dissolved in the minimum volume of cold ethanol and reprecipitated several times by addition of ether to give a colourless powder. The powder recrystallized from propan-1-ol as fine colourless needles that became sticky on exposure to air; on keeping in a desiccator over $CaCl_2$, it gave *N-acetyl-S-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine monohydrate* as a colourless powder (2·8g., 87%), m.p. 108–112° (decomp.) after softening at 54–60° (Found: C, 46·4; H, 7·25; N, 8·5; S, 9·8. $C_{13}H_{24}N_2O_6S$ requires C, 46·4; H, 7·2; N, 8·3; S, 9·5%), $[\alpha]_D^{25}$ +1·6° (*c* 1 in methanol). The *anhydrous* compound (III), m.p. 126–130° (decomp.) after softening at 98–100°, was obtained after drying the powder at 62°/15mm. Hg over P_2O_5 for 16hr. (Found: C, 49·5; H, 7·3; N, 9·05; S, 10·45. $C_{13}H_{22}N_2O_5S$ requires C, 49·05; H, 7·0; N, 8·8; S, 10·05%). An aqueous solution of this compound did not react with bromine or iodine, showing that it contained no ethylenic bond or free thiol group.

N-Acetyl-S-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine (V). The preceding experiment was repeated with arecaidine (1·4g.) in place of arecoline and the mixture was analysed by chromatography in solvents (*b*) and (*c*) after 4, 6 and 30hr. No reaction was detected after 4 and 6hr. and traces of compound (V) (for R_F values see Table 1) were formed after 30hr. Duplicate samples (10mg.) of compound (III) were dissolved in 5ml. each of (i) M-NaOH, (ii) M-HCl and (iii) saturated aq. $NaHCO_3$. One sample of each group was kept at 25° and the other was heated at 80°. Chromatography in solvents (*b*) and (*c*) after 1hr. revealed that no reaction occurred in mixtures (iii), whereas mixture (i) at 80° and mixtures (ii) gave a complex mixture of unidentified products. Mixture (i) at 25° formed one major product having R_F 0·33 and R_F 0·46 in solvents (*b*) and (*c*) respectively. This product gave positive reactions with reagents (ii) and (iv) and a negative reaction with reagent (iii); it thus appeared to be compound (V), which was synthesized as follows. A solution of compound (III) (0·9g.) in 2M-NaOH (5ml.), after 3hr. at 25°, was treated with 2M-HCl (5ml.), the resulting solution evaporated *in vacuo* and the residue extracted with methanol (3×5ml.). The extracts were combined and evaporated *in vacuo* and the residual solid was recrystallized from ethanol to give colourless needles (0·4g.) that became sticky on exposure to air. On storage in a desiccator over $CaCl_2$ it resolidified to give *N-acetyl-S-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine monohydrate* as a colourless powder, m.p. 188–192° (decomp.) after softening at 102–104° [Found: C, 44·5; H, 6·9; N, 8·9; S, 10·2; H_2O (by wt. loss after drying at 62°/15mm. Hg over P_2O_5 for 16hr.), 5·2. $C_{12}H_{22}N_2O_5S$ requires: C, 44·7; H, 6·9; N, 8·7; S, 9·9; H_2O, 5·6%], $[\alpha]_D^{25}$ +1·7° (*c* 1 in methanol). On storage in a desiccator over $CaCl_2$ at 23° for 2–3 days the

compound decomposed into a brown gum that, by chromatography in solvent (*b*), was a mixture of at least four unidentified substances.

N-Acetyl-S-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine methyl ester (VI). A solution of compound (III) (5g.) in methanol (10ml.) was treated with diazomethane in ether, in portions, until the yellow colour persisted for at least 1hr. After 16hr. the mixture was evaporated to a viscous yellow oil. Chromatography revealed that this contained one major component having R_F 0·77 and R_F 0·64 in solvents (*a*) and (*b*) respectively, and giving positive reactions with reagents (ii) and (iv) and a negative reaction with reagent (iii). Attempts to purify the oil by distillation at 0·01mm. Hg (which resulted in extensive decomposition), by crystallization, or by preparation of solid derivatives (hydrochloride, hydrobromide, perchlorate, reineckate, picrate) were unsuccessful. A chromatographically homogeneous specimen was obtained by elution with methanol of strips cut off the appropriate R_F region from paper chromatograms on which the oil (1g.) was applied as streaks and which were developed in solvent (*b*). Evaporation of the solvent *in vacuo* gave a colourless oil (0·84g.). This oil (0·1g.), after hydrolysis with 2M-NaOH (0·2ml.) at 60° for 1hr., gave compound (V) and a product having R_F 0·09 and R_F 0·44 in solvents (*a*) and (*b*) respectively. This product was eluted with methanol from chromatograms developed in solvent (*b*). Evaporation *in vacuo* of the methanolic eluate and treatment of the residual gum with diazomethane in ether converted this product into compound (VI). The product gave positive reactions with reagents (ii) and (iv) and a negative reaction with reagent (iii); it was probably *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine methyl ester (VII).

Treatment of a solution of compound (V) (0·1g.) in methanol (2ml.) with diazomethane in ether, as described above, gave an oil that showed properties similar to those described for compound (VI).

Acute toxicities. Arecoline, by a single intraperitoneal injection in water (0·1ml.), showed LD_{50} of approx. 40mg./kg. in male adult Chester Beatty rats. A dose of 80mg./kg. killed all animals within 2hr., after violent convulsions, salivation, inhibition of spontaneous activity, and capillary dilatation around the eyes, nose, mouth and feet. The Merck Index (1968) reports a lethal dose of 100mg./kg. by subcutaneous injection in mice. Arecaidine hydrochloride, by a single intraperitoneal injection in water (0·1ml.), was less toxic; LD_{50} was approx. 0·8g./kg. in male adult Chester Beatty rats, death occurring within 48hr. of injection. The typical parasympathomimetic effects described for arecoline were absent, even after administration of 1·6g./kg.

Spectra. (*a*) Arecoline (I). (i) The n.m.r. spectrum (in $CDCl_3$) showed 13 protons: one vinylic proton (multiplet, 2·90–3·2τ), three ester methyl protons (singlet, 6·3τ), two 2-methylene protons (multiplet, 6·67–7·10τ), three *N*-methyl protons (singlet, 7·62τ) and four 5,6- dimethylene protons (multiplet, 7·34–7·80τ). (ii) The i.r. spectrum (liquid film) showed absorption bands at 1715cm.$^{-1}$ (strong) (C=O) and 1267cm.$^{-1}$ (strong) (C—O). (iii) The u.v. spectrum (0·1mM solution in 5mM-phosphate buffer, pH8·0) showed $\lambda_{max.}$ at 216nm. (ϵ 9200).

(*b*) *N*-Acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine (III). (i) The n.m.r. spectrum (in $CDCl_3$) showed 22$\pm$2 protons as follows: one carboxylic acid proton as a broad peak (0·53–1·22τ), one N-H proton as a broad peak (5·15–5·67τ), three ester methyl protons (singlet, 6·22τ), a singlet (7·94τ) containing *N*-acetyl methyl protons and *N*-methyl protons, and a broad envelope of peaks (6·1–8·2τ) containing remaining protons. (ii) The i.r. spectrum (KBr disk) showed absorption bands at 3400cm.$^{-1}$ (strong, broad) (OH), 2700–2500cm.$^{-1}$ (weak, broad) (OH or NH^+), 1735 and 1728cm.$^{-1}$ (strong) (C=O), 1964cm.$^{-1}$ (strong) (amide I), 1610cm.$^{-1}$ (moderate) (CO_2^-, asymmetric mode), 1542cm.$^{-1}$ (moderate) (amide II) and 1375cm.$^{-1}$ (moderate) (CO_2^-, symmetric mode). (iii) The u.v. spectrum (0·1mM solution in 5mM-phosphate buffer, pH8·0) showed $\lambda_{max.}$ at 202nm. (ϵ 5500).

RESULTS

Metabolism of arecoline. Arecoline hydrochloride was administered by daily injection of 20mg./kg. body wt. (total dose, 0·96g.) to 12 female rats during 3 weeks. The parasympathomimetic effects described above subsided after about 30min. Animals that died (one each on days 8, 13 and 16; two each on days 6 and 18) were replaced. Control animals were injected daily with water (0·2ml.). The urine from each group was separated from the toluene, adjusted to pH5·0 with acetic acid, filtered (Ford's clarifying pad, grade F.C.B.), and the filtrate continuously extracted for 20hr. with chloroform containing 5% (v/v) of ethanol. The organic phase and the toluene previously separated were combined and evaporated *in vacuo*. The residue was examined by chromatography in solvent (*b*). The extract from the urine of arecoline-treated animals contained two substances that did not appear in the control specimen. One substance (metabolite A), having R_F 0·38–0·41 and giving a pink colour with reagent (iv), appeared to be identical with arecaidine (IV); the other substance (metabolite B), having R_F 0·28–0·33 and giving positive reactions with reagents (ii) and (iv), appeared to be identical with compound (V). The residue from the treated animals was extracted with methanol (3 × 20ml.), the extracts were combined and evaporated to 3ml., and 1ml. was applied as streaks on sheets of Whatman 3MM paper. The two metabolites were eluted with aq. 60% ethanol from the appropriate strip regions of the chromatograms developed in solvent (*b*). Chromatography of the concentrated aqueous ethanolic eluates in solvents (*a*)–(*d*) showed that metabolites A and B were chromatographically indistinguishable from arecaidine and compound (V) respectively (for R_F values see Table 1). By visual comparison of the intensities of spots on developed chromatograms containing known amounts of the reference compounds with those obtained from 0·2ml. of the methanolic extract (representing 64mg. of arecoline given), 0·2–1% of the dose was converted into compound (V) and 1–3% into compound (IV). The remainder (2ml.) of the methanolic extract was used to compare the

patterns of metabolites in the urine of animals receiving arecoline in the drinking water and of animals receiving arecaidine by injection.

The aqueous urinary fraction from the chloroform–ethanol extraction was adjusted to pH 3 with 2M-hydrochloric acid and shaken with charcoal (8g.). The charcoal was filtered off, washed free of Cl^- ions with water, shaken with methanol containing 5% (v/v) of aq. ammonia (sp.gr. 0·88), filtered off, the filtrate evaporated *in vacuo* and the residue examined by chromatography as described above. Arecaidine and compound (V) were found in trace amounts.

The urine from 12 female adult Chester Beatty rats that received arecoline hydrochloride in the drinking water over a period of 10 days (approx. total dose 4·8g.) was extracted continuously with chloroform–ethanol and the extracted material dissolved in methanol (24ml.) as described above. The residue obtained by evaporation of the methanolic extract (2ml.) was compared by chromatography in solvents (*b*) and (*c*) with the residues obtained similarly from the corresponding methanolic extracts (2ml. each) described in the foregoing experiment. Visual comparison of spot intensities on the chromatograms indicated that the animals receiving arecoline hydrochloride in the drinking water excreted in the urine (*a*) about two- to three-fold more arecaidine and (*b*) about four- to ten-fold less of compound (V) than animals that received the drug by injection.

Metabolism of arecaidine. Arecaidine hydrochloride was administered by daily intraperitoneal injections of 250mg./kg. body wt. (total dose 12·0g.) to 12 female rats during 3 weeks. The drug produced none of the immediate after-effects described for arecoline hydrochloride. Two animals that died (one each on days 11 and 17) were replaced. Control animals received daily injections of 0·2ml. of water. The test and control urine samples were fractionated into chloroform–ethanol and charcoal fractions and examined by chromatography as described for arecoline metabolism. Two substances chromatographically indistinguishable from arecaidine and compound (V) were found in both fractions. The chloroform–ethanol fraction was evaporated to dryness *in vacuo* and the residual thick oil stored at 4°. This deposited colourless prisms (1·5g.), mainly of inorganic salts (no m.p. below 320°) contaminated with an unidentified substance that gave a positive naphtharesorcinol test for glucuronides. The mother liquor was evaporated *in vacuo* and the residue fractionally recrystallized from hot aq. 80% (v/v) ethanol to give two further crops of inorganic salts, then arecaidine (20mg.) as colourless needles, m.p. and mixed m.p. 228–229° (decomp.) after drying at 100°/15mm. Hg over phosphorus pentoxide for 2hr. (Found: N, 9·5. Calc. for $C_7H_{11}NO_2$: N, 9·9%). The filtrate was combined with the charcoal fraction and the combined solutions were evaporated *in vacuo*. The residual gum was shaken with ether (3 × 50ml.), the ethereal extracts were combined, dried (over anhydrous sodium sulphate) and evaporated to yield an oil, which solidified. The solid was recrystallized from light petroleum to yield benzoic acid (150mg.) as colourless needles, m.p. and mixed m.p. 122°. The ether-insoluble residue was dissolved in water (3ml.) and applied as streaks to sheets of Whatman 3MM paper, and the strip regions corresponding to arecaidine and compound (V) were extracted with hot methanol from the dried chromatograms after development in solvent (*b*). From visual comparison of the intensities of spots on developed chromatograms as described above, arecaidine was excreted unchanged to the extent of 2–4% and compound (V) to the extent of 0·1–1%. The remainder of the eluted materials was treated with diazomethane in ether as described above. Chromatography of the products in solvents (*a*)–(*d*) showed that this treatment converted arecaidine into arecoline and compound (V) into compound (VI).

Reaction between arecoline or arecaidine and glutathione with and without added liver preparations. The following incubation mixtures (in triplicates) contained (per ml.) glutathione (5μmoles), arecoline or arecaidine (12·5μmoles), sodium phosphate buffer, pH 6·8 (100μmoles of phosphate), and, where used, whole liver homogenate or dialysed supernatant (dialysed as described by Boyland & Chasseaud, 1967) from 50mg. wet wt. of liver: (1) arecaidine + glutathione; (2) arecaidine + glutathione + dialysed liver supernatant from (*a*) mouse, (*b*) rat, (*c*) hamster and (*d*) duck; (3) arecaidine + glutathione + whole rat liver homogenate; (4) arecoline + glutathione; (5) arecoline + glutathione + rat liver homogenate. Six similar mixtures, with the alkaloids omitted, served as controls. After 40min. at 37°, each mixture was treated with an equal volume of 4% (w/v) sulphosalicylic acid to lower the pH to approx. 2, to stop the reaction (see Fig. 1) and precipitate proteins. Unchanged glutathione was determined by iodate titration in the presence of iodide and sodium starch glycollate (Woodward & Fry, 1932). The results, expressed as percentage molar loss of thiol (test minus control), were: (1) 2·0, 5·2, 5·2; (2*a*) 0·8, 2·0, 3·9; (2*b*) 2·3, 2·5, 4·6; (2*c*) 1·8, 2·0, 2·6; (2*d*) 2·9, 3·2, 4·0; (3) 1·4, 1·6, 2·0; (4) 88, 89, 92; (5) 28, 32, 36.

Effect of pH *on the reaction between arecoline or arecaidine and thiol.* Glutathione (5μmoles) or *N*-acetyl-L-cysteine (5μmoles) was dissolved in each of the following (1ml.): 2% (w/v) sulphosalicylic acid (approx. pH 2), 0·2M-sodium acetate buffer, pH 4·0 and 5·0, and 0·2M-sodium phosphate buffer, pH 6·0, 6·5, 7·0, 7·4 and 8·0. The solutions

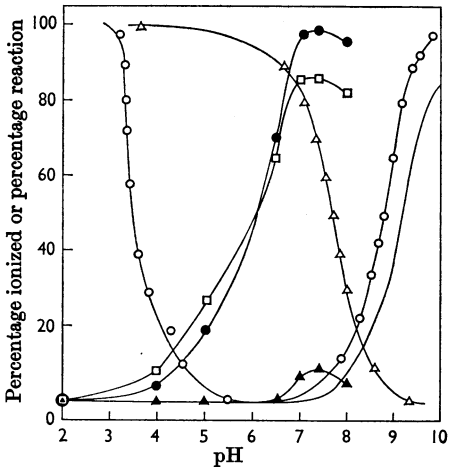


Fig. 1. Arecoline or arecaidine (50ml. of a 0·01M solution) was titrated with M-hydrochloric acid; a similar solution of arecoline hydrochloride or 50ml. of 0·2mM-arecaidine hydrochloride solution was titrated with M- or 0·1M-potassium hydroxide respectively. Solutions were made up in deionized carbon dioxide-free water and titrations were done at 25°. At the pH values <2·8, 5·4–6·8 and >9·9, arecaidine formed the quaternary ammonium ion (net charge, +1), the zwitterion (net charge, 0) and the carboxylate anion (net charge, −1) respectively. For the reactions between glutathione or *N*-acetyl-L-cysteine and arecoline or arecaidine, solutions of the thiol (5μmoles) and the alkaloid (25μmoles) in the appropriate buffer (1ml.) were incubated at 37° for 20min. and the thiol contents determined. Titration curves: glutathione (——; cf. Benesch & Benesch, 1955), arecoline (△) and arecaidine (○). Also shown is the effect of pH on the reactions between arecoline and *N*-acetylcysteine (□), arecoline and glutathione (●) and arecaidine and glutathione (▲). For further details see the text.

were incubated with and without arecoline (25μmoles) or arecaidine (25μmoles) at 37° for 20min., treated with 4% (w/v) sulphosalicylic acid (1ml.) and the thiol contents determined as described above. The results are shown in Fig. 1; points are the means of triplicate determinations with deviations of ±2–5%, except for those obtained from the mixtures containing arecaidine and glutathione, which showed deviations of ±6–20%. No reaction occurred between *N*-acetyl-L-cysteine and arecaidine under the conditions described.

Kinetics of the reaction between arecoline or arecaidine and glutathione. The following mixtures were incubated at 37°. Glutathione (5μmoles) and arecoline [at three concentrations: (*a*) 5·05, (*b*) 6·25 and (*c*) 25μmoles/ml. of 0·2M-sodium phosphate buffer, pH7·0]; *N*-acetyl-L-cysteine (5μmoles) and arecoline [at three concentrations: (*d*) 5·05, (*e*) 6·25 and (*f*) 25μmoles/ml. of the same buffer]; two control mixtures containing (*g*) glutathione (5μmoles/ml. of buffer) or (*h*) *N*-acetyl-L-cysteine (5μmoles/ml. of buffer). At 2min. intervals during 20min., 1ml. samples of each mixture were treated with 4% (w/v) sulphosalicylic acid (1ml.) and the thiol content was determined by titration with 0·166mM-iodate in the presence of iodide and sodium starch glycollate as described above. The kinetic order of each reaction, obtained by the differential method (Glasstone, 1947), was 1·8–1·9 for reactions (*a*), (*b*), (*d*) and (*e*) and the plots of time versus $\log(a-x/b-x)$ were linear, indicating second-order kinetics with $k=0{\cdot}103\pm0{\cdot}02$, $0{\cdot}101\pm0{\cdot}02$, $0{\cdot}085\pm0{\cdot}018$ and $0{\cdot}083\pm0{\cdot}021$.mole^{-1}sec.$^{-1}$ respectively. The kinetic orders for reactions (*c*) and (*f*) were 1·05 and 1·01 respectively, and the plots of time versus $\log(a-x)$ were linear, indicating first-order kinetics with $k=4{\cdot}91\pm0{\cdot}05\times10^{-4}$ and $2{\cdot}20\pm0{\cdot}04\times10^{-4}$sec.$^{-1}$ respectively. In the calculations, a and b were the initial concentrations, in moles l.$^{-1}$, of thiol and arecoline respectively, and x was moles l.$^{-1}$ that had reacted at time t; $b-x$ was obtained by assuming a 1:1 molar ratio in the reaction between arecoline and thiol.

Arecoline was replaced with arecaidine in the reactions (*c*) and (*f*) (above), and the resulting mixtures were analysed after 20, 35, 45, 80 and 120min. The results, expressed as percentage molar loss of thiol by reaction with arecaidine, were 6·7, 7·4, 10·0 and 8·8 in mixture (*c*) and none in mixture (*f*). These erratic values were probably due to (i) the slowness of the reactions, (ii) non-specific thiol reactions and (iii) the relative instability of the thiol–arecaidine products (cf. the instability of compound V).

Determination of degree of ionization and pK_a of arecoline and arecaidine. Arecoline, which is a colourless liquid when freshly distilled (b.p. 94°/12mm. Hg), becomes yellow to deep red on storage. For these experiments, arecoline was twice redistilled before use; arecaidine hydrochloride was recrystallized and dried as described above. Carbonate-free potassium hydroxide was prepared as described by Albert & Serjeant (1962). All solutions were made up in deionized carbon dioxide-free water, double-distilled in an all-glass apparatus. All titrations were performed at 25° in an atmosphere of purified nitrogen with a Pye universal pH-meter and a Pye combined glass–calomel electrode. Arecoline (50ml. of 0·01M solution) was titrated with M-hydrochloric acid in 0·05ml. portions and arecaidine hydrochloride (50ml. of 2mM solution) with 0·1M-potassium hydroxide in 0·10ml. portions. The ionization and pK_a values were calculated as described by Albert & Serjeant (1962). The results, shown in Fig. 1, have been corrected for dilution by added titrant. The pK_a of arecoline was $7{\cdot}69\pm0{\cdot}03$, and the pK_a values of arecaidine were $3{\cdot}62\pm0{\cdot}06$ (carboxyl group) and

$8{\cdot}82 \pm 0{\cdot}06$ (*tert.*-amino group). The corresponding ionization constants were $2{\cdot}04 \times 10^{-8}$, $2{\cdot}4 \times 10^{-4}$ and $1{\cdot}5 \times 10^{-9}$. These results were also obtained from the titration of arecoline hydrochloride (50ml. of 0·01M solution) with M-potassium hydroxide or of arecaidine (50ml. of 0·01M solution) with M-hydrochloric acid or M-potassium hydroxide.

Reaction between arecaidine and N-*acetyl*-L-*cysteine.* A solution of *N*-acetyl-L-cysteine (0·1g.) and arecaidine (0·1g.) in 0·2M-phosphate buffer, pH 7·0 (0·5ml.), was heated at 37° and 0·01ml. samples were analysed by chromatography in solvents (*a*), (*b*) and (*c*) after 2, 8 and 25hr. No reaction occurred after 2 and 8hr. and a trace of compound (V) was formed after 25hr.

Failure of arecoline or arecaidine to react with nucleic acid bases. Duplicate 0·02M solutions (0·5ml.) of each of the following bases in 0·1M-phosphate buffer, pH 7·5, were treated with a solution of 0·1M-arecoline (0·5ml.) in the same buffer: cytosine, cytidine, cytidylic acid, deoxycytidylic acid, thymidylic acid, deoxyuridylic acid, adenosine, adenylic acid and guanylic acid. Control mixtures (in duplicate) contained the solution of each base (0·5ml.) and buffer (0·5ml.). One group of the resulting solutions was kept at 25° and the other was heated at 80°. Samples (0·1ml.) of each solution were applied as streaks on sheets of Whatman 3MM paper after 2hr. and 6hr. and developed in solvents (*b*) and (*c*). Compounds were located by scanning the dried chromatograms under u.v. light; they were eluted off the appropriate strip regions with 1mM-phosphate buffer and their u.v. spectra determined. Samples (0·01ml.) of each reaction mixture were diluted with water after 1, 5 and 22hr. and their u.v. spectra determined. A comparison of the results obtained from the test and control samples did not reveal any reaction between arecoline and any of the bases. The reactions were repeated with arecaidine in place of arecoline and the following bases: cytosine, uridine, thymidine, adenosine and guanosine in 0·1M-acetate buffer, pH 4·5, and in 0·1M-phosphate buffer, pH 7·5. No reactions were detected.

DISCUSSION

The widespread habit, especially in the Orient, of chewing quids composed of betel nut and lime, with or without tobacco, is associated with oral cancers (Muir, 1967). Extracts of betel-nut–tobacco quid are weakly carcinogenic to mice (Muir & Kirk, 1960). Of the six known alkaloids of betel nut, those with known structure (arecoline, arecaidine, guvacine and guvacoline) are derivatives of 1,2,5,6-tetrahydropyridine, i.e. they all contain a Δ^3-ethylenic bond. Reasons for believing that one or more of these alkaloids might be carcinogenic have been reviewed (Boyland, 1968).

The present results show that the Δ^3-ethylenic bonds of arecoline and arecaidine react with thiol groups *in vivo* and *in vitro*. Arecoline readily reacts with *N*-acetyl-L-cysteine and with glutathione in neutral aqueous solution at 37°; arecaidine reacts much more slowly. The parasympathomimetic effects immediately produced when rats are dosed with arecoline, compared with the absence of such effects when the animals receive comparable or larger doses of arecaidine, may be due to rapid reaction of arecoline with thiol enzymes.

The addition of the thiol group of glutathione across the Δ^3-ethylenic bond of arecoline is apparently not enzyme-catalysed, since various liver preparations did not increase the rate of the reaction at pH 6·8 and 37°. The observed decreased rate was probably due to the enzymic hydrolysis of arecoline into arecaidine (Nieschulz & Schmersahl, 1968), which reacts more slowly with glutathione (see Fig. 1). The simultaneous occurrence of two competing reactions makes the present results difficult to interpret. The reaction between glutathione and arecoline is rapid and maximal at neutral pH, the rate decreasing rapidly as the pH is lowered, does not occur below pH 2·5 and falls to about 95% of the maximal rate at pH 8·0. At pH 7·0 and 37° the reaction between glutathione and arecoline in approximately equimolar concentrations was of second-order with k = approx. 10^{-1}mole^{-1}sec.$^{-1}$; when the alkaloid was present in fourfold excess the reaction was pseudo-unimolecular first order with $k = 4{\cdot}91 \pm 0{\cdot}05 \times 10^{-4}$sec.$^{-1}$. The reactions of arecoline with *N*-acetyl-L-cysteine were qualitatively similar to the corresponding reactions with glutathione, i.e. similar effects of pH (see Fig. 1) and relative concentrations of the alkaloid with respect to thiol were observed; quantitatively, the pseudo-unimolecular first-order specific reaction rate was $2{\cdot}20 \pm 0{\cdot}04 \times 10^{-4}$sec.$^{-1}$ and the second-order specific reaction rate was approx. 8×10^{-2}l.mole^{-1} sec.$^{-1}$.

At the pH region (pH 7·0–7·4) of maximal reaction between arecoline and glutathione (Fig. 1) approx. 15–30% of the arecoline and 100% of the thiol group were un-ionized; with increasing alkalinity or acidity, a corresponding increase in the percentage thiol or alkaloid ionized caused a corresponding decrease in the extent of reaction.

The mechanism of the addition of thiols to the Δ^3-ethylenic bond of arecoline is complex. If it were a nucleophilic addition (Ad_N) involving attack by thiol on an activated ethylenic bond, the reaction should be facilitated by protonation of the ester carbonyl group in acid and by thiolate anion formation in alkali, and the nucleophilic thiol group would be directed to the 4-position of the tetrahydropyridine ring. Such a directional effect has

been observed. If a free-radical mechanism operated, ionization of either reactant in acid or alkali would hinder the reaction, but the directive effects would be less marked. In either mechanism, reversibility of the reaction would increase in acid or alkali (the so-called principle of microscopic reversibility; see Ingold, 1951) and the reaction would be hindered by ester hydrolysis to give the much less reactive arecaidine (see Fig. 1).

The pK_a of arecoline was 7·69. Arecaidine had pK_a 3·62 for the carboxyl group and pK_a 8·82 for the *tert.*-amino group. The titration curve (Fig. 1) shows that the *tert.*-amino group of arecaidine is completely ionized below pH 2·8; arecaidine exists in the neutral form, probably as the zwitterion, between pH 5·4 and pH 6·8, and exclusively as the carboxylate anion above pH 9·9. Arecoline (Fig. 1) exists as the quaternary ammonium ion below pH 3·8 and as the free base above pH 9·3. The stronger basicity of the *tert.*-amino group of arecaidine as compared with that of arecoline results from the greater electronegativity of the substituted acrylic ester group of arecoline as compared with the substituted acrylate anion of arecaidine.

The mercapturic acid excreted in the urine of rats dosed with arecoline (I) or arecaidine (IV) was identical in its chromatographic behaviour with *N*-acetyl-*S*-(3-carboxy-1-methylpiperid-4-yl)-L-cysteine (V), which was prepared by cold alkaline hydrolysis of *N*-acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine (III). Compound (III) or (V), on treatment with diazomethane, gave *N*-acetyl-*S*-(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine methyl ester (VI). The i.r. spectrum of compound (III) showed absorption bands near 3300 cm.$^{-1}$ and 3050 cm.$^{-1}$ together with bands at 1654 cm.$^{-1}$ (amide I) and 1542 cm.$^{-1}$ (amide II) that are consistent with the presence of a secondary amide group. The doublet at 1735/1728 cm.$^{-1}$ can be assigned to the ester and carboxylic acid carbonyl groups respectively; bands at 3400 cm.$^{-1}$ and 2700–2500 cm.$^{-1}$ would then be assigned to OH (associated). However, the absorption due to OH (associated) arising from the carboxyl group should be centred near 3000 cm.$^{-1}$. Assuming that the doublet near 1730 cm.$^{-1}$ arises solely from the ester carbonyl group, the bands at 1610 cm.$^{-1}$ and 1375 cm.$^{-1}$ can be assigned to the antisymmetric and symmetric stretching modes of the CO_2^- group respectively, which would indicate that compound (III) exists partly in the zwitterionic form (compound IIIa). The broad band at 3400 cm.$^{-1}$ would then be attributed to the OH stretching mode of water of crystallization. The n.m.r. spectrum of compound (III) showed 22 ± 2 protons comprising one carboxylic acid proton, one N-H proton, three ester methyl protons, three *N*-acetyl methyl protons, three *N*-methyl protons and a broad envelope of peaks containing the remaining protons that could not be accurately assigned. The addition of the thiol group of *N*-acetyl-L-cysteine across the Δ^3-ethylenic bond of arecoline or arecaidine creates two new asymmetric centres and thus four optical isomers are possible. No attempt was made to determine the steric course of the reaction, but the apparent chromatographic homogeneity and small optical activity of the products indicate some stereospecificity.

The reactions of arecoline and arecaidine with thiol groups show that they are biological alkylating agents; this is a feature of many chemical carcinogens either with or without metabolic activation (reviewed by Miller & Miller, 1966). This is, however, no proof of carcinogenic potential, although it has been shown that such interactions with thiol groups can lead to uncontrolled cell proliferation and cancer (Harrington, 1967). In aqueous media, no evidence of reaction was seen between arecoline or arecaidine and the nucleic acid bases, although such reactions may occur *in vivo*.

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REFERENCES

Albert, A. & Serjeant, E. P. (1962). *Ionization Constants of Acids and Bases*, pp. 9 *et seq.* London: Methuen and Co. Ltd.

Bartley, W. (1954). *Biochem. J.* **56**, 379.

Benesch, R. E. & Benesch, R. (1955). *J. Amer. chem. Soc.* **77**, 5877.

Boyland, E. (1968). *Planta Med.* Suppl., p. 13.

Boyland, E. & Chasseaud, L. F. (1967). *Biochem. J.* **104**, 95.

Glasstone, S. (1947). *Textbook of Physical Chemistry*, 2nd ed., p. 1067. New York: D. Van Nostrand Co. Inc.

Harrington, J. S. (1967). *Advanc. Cancer Res.* **10**, 247.

Ingold, C. K. (1951). *Structure and Mechanism in Organic Chemistry*, pp. 216, 651. London: G. Bell and Sons Ltd.

Knight, R. H. & Young, L. (1958). *Biochem. J.* **70**, 111.

Miller, E. C. & Miller, J. A. (1966). *Pharmacol. Rev.* **18**, 805.

Muir, C. S. (1967). In *The Prevention of Cancer*, pp. 75, 81. Ed. by Raven, R. W. & Roe, F. J. C. London: Butterworths Scientific Publications.

Muir, C. S. & Kirk, R. (1960). *Brit. J. Cancer*, **14**, 597.

Nieschulz, O. & Schmersahl, P. (1968). *Arzneimittel Forsch.* **18**, 222.

Wohl, A. & Johnson, A. (1907). *Ber. dtsch. chem. Ges.* **40**, 4712.

Woodward, G. E. & Fry, E. G. (1932). *J. biol. Chem.* **97**, 465.