The Binding of Radioactive Label from Labelled Phenacetin and Related Compounds to Rat Tissues *in vivo* and to Nucleic Acids and Bovine Plasma Albumin *in vitro*

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1. *acetyl*-³H- and *ethyl*-¹⁴C-labelled derivatives of phenacetin and related compounds are described. 2. Radioactive label from the *ethyl*-¹⁴C-labelled derivatives of 4-nitrophenetole, 4-phenetidine and phenacetin binds *in vitro* to various extents to bovine plasma albumin, salmon sperm DNA and yeast RNA; the extent of binding is increased in the presence of a rat liver microsomal hydroxylating system and further increased when the microsomal enzymes are induced by prior treatment of rats with 3-methylcholanthrene. 3. The ratios of the bound radioactive labels *in vitro* from [*ethyl*-¹⁴C]phenacetin, *N*-acetoxy[*ethyl*-¹⁴C]phenacetin, [*acetyl*-³H]-phenacetin and [*diacetyl*-³H]*N*-acetoxyphenacetin per g-atom of DNA P, RNA P and per mol of protein in the absence of the microsomal system are approximately 1:60:11:863, 1:68:41:1835 and 1:88:713:2399 respectively. 4. Radioactive label from labelled phenacetin binds *in vitro* to all tissues examined, including the spleen, intestines, kidney and bladder; about 80% of the radioactivity bound to the liver is concentrated in the RNA and proteins. 5. Comparison of the relative extents of binding of radioactive label derived from equimolar amounts of labelled phenacetin, ethanol or acetate shows that the incorporation of labelled C₂ units into tissues and biological macromolecules *in vivo* and *in vitro* may account for only a part of the total bound radioactive label derived from phenacetin and not at all from the incorporation of radioactive acetate into nucleic acids. 6. Some implications of these findings are discussed.

Phenacetin is a methaemoglobinogenic and nephrotoxic agent, and phenacetin abuse may be a contributory cause of cancers of the bladder and kidney in humans (Wuhrmann & Jasinski, 1955; Angervall, Bengtsson, Zetterlund & Zsigmond, 1969). The present paper describes the effect of metabolism on the binding properties of phenacetin *in vivo* and *in vitro*.

tate were administered as solutions in water (0.2 ml). The animals were starved for 8 h before and during the experiment. Water was available *ad lib*.

Melting points

The melting points (uncorrected) of the radioactive compounds were the same as those of the corresponding unlabelled pure compounds and were unchanged on admixture of the two compounds.

MATERIALS AND METHODS

Measurement of radioactivity

This was performed on a Packard Tri-Carb liquid-scintillation spectrometer and represents counts at least 50% above background corrected for quenching by the addition of an internal standard.

Animals

Compounds were administered to 6-7-week-old male Chester Beatty rats (mean body wt. 180 g) by gavage; phenacetin was administered as a 10% (w/v) suspension in aq. 10% (w/v) gum acacia, and ethanol and sodium ace-

Chemicals

DNA (from salmon testes) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; highly polymerized yeast RNA was from British Drug Houses Ltd., Poole, Dorset, U.K.; bovine plasma albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.; NADP⁺, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were from Boehringer Corp. (London) Ltd., London W.5, U.K. Ribonuclease A was obtained from Sigma Chemical Co. and deoxyribonuclease I (D) from Worthington Biochemical Corp., Freehold, N.J., U.S.A. [¹⁴C]Ethyl iodide (specific radioactivity, 61 mCi/mmol), [³H]acetic anhydride (specific radioactivity 100 mCi/mmol), sodium [³H]acetate (specific radioactivity

100mCi/mmol) and [1-³H]ethanol (specific radioactivity 250mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other chemicals not described below were of commercial origin.

4-Nitrophenetole. A solution of 4-nitrophenol (14g), diethyl sulphate (16g) and sodium ethoxide (prepared from 5g of sodium) in ethanol (200ml) was filtered after 16h at 150°C in a sealed tube, the residue was washed with hot ethanol (50ml) and the combined filtrate and washing were evaporated to dryness *in vacuo*. Recrystallization of the residue from aqueous ethanol afforded 4-nitrophenetole as buff needles, (14.8g; 89% yield), m.p. and mixed m.p. 57°C.

4-Nitro[ethyl-1-¹⁴C]phenetole. A solution of 4-nitrophenol (4.2g) and sodium ethoxide (prepared from 0.7g of sodium) in ethanol (60ml) containing [1-¹⁴C]ethyl iodide (4.7g; 1mCi) was heated in a sealed tube at 120°C for 8h. T.l.c. on silica gel plates in benzene–light petroleum (b.p. 40–60°C) (1:3, v/v) showed that the nitrophenol (R_F 0.05) was almost completely converted into 4-nitrophenetole (R_F 0.6). The mixture was evaporated *in vacuo* to approx. 10ml, diluted with 1M-NaOH (50ml) at 0°C and the precipitate filtered off, washed with water (2ml) and recrystallized from aqueous ethanol to give buff needles of 4-nitro[ethyl-1-¹⁴C]phenetole (4.6g; 92% yield; specific radioactivity 33mCi/mol), m.p. 57°C (Found: C, 57.3; H, 5.4; N, 8.3. Calc. for C₈H₉NO₃: C, 57.5; H, 5.4; N, 8.4%).

[ethyl-1-¹⁴C]4-Phenetidine. A solution of NaBH₄ (2g) in water (25ml) was added cautiously to a mixture of 10% palladium charcoal (0.2g) in water (25ml) under N₂. The mixture was stirred and treated with a solution of 4-nitro[ethyl-1-¹⁴C]phenetole (1.9g; 375μCi) in ethanol (50ml), when an exothermic reaction ensued. The nitro compound was added at a rate sufficient to maintain the reaction temperature at 40–45°C. The mixture was filtered after 5h, the residue was washed with ethanol (25ml), and the filtrate and washing were combined and acidified to pH 2.0 with 6M-HCl at 0°C, evaporated *in vacuo* to approx. 20ml, made alkaline to pH 9.0 with 10M-NH₃ and extracted with ether (3×50ml). The ether extracts were dried (over Na₂SO₄) and evaporated *in vacuo*, and the residual oil was dissolved in ethanol (5ml) and treated with dry HCl at 0°C to give a precipitate, which was recrystallized from ethanol to yield colourless needles of [ethyl-1-¹⁴C]4-phenetidine hydrochloride (1.8g; 92% yield; specific radioactivity 33mCi/mol), m.p. 234°C (Found: C, 55.5; H, 7.1; Cl, 20.1; N, 8.0. Calc. for C₈H₁₂ClNO: C, 55.4; H, 7.0; Cl, 20.4; N, 8.1%).

[ethyl-1-¹⁴C]Phenacetin. A solution of [ethyl-1-¹⁴C]4-phenetidine hydrochloride (1.7g; 0.33mCi) in dry redistilled pyridine (10ml) and acetic anhydride (4ml) was diluted with ice-water (50ml) after 16h at 4°C and the precipitate recrystallized from ethanol to give colourless needles of [ethyl-1-¹⁴C]phenacetin (1.65g; 95% yield; specific radioactivity 33mCi/mol), m.p. 138°C (Found: C, 66.9; H, 7.3; N, 7.9. Calc. for C₁₀H₁₃NO₂: C, 67.0; H, 7.3; N, 7.8%).

[³H-acetyl]Phenacetin. As described for the ethyl-¹⁴C-labelled analogue, a solution of 4-phenetidine hydrochloride (2.15g) and [³H]acetic anhydride (1.02g; 13.5mCi) in pyridine (10ml) gave colourless needles of [³H]-phenacetin (1.7g; 95% yield; specific radioactivity 470mCi/mol), m.p. 138°C (Found N, 7.8%).

N-Hydroxy-[ethyl-¹⁴C]4-phenetidine. This was prepared by a modification of the method of Rising (1904). A solution of 4-nitro[ethyl-¹⁴C]phenetole (1.0g; 197μCi) in ethanol (10ml) was shaken at 65°C with zinc dust (1.0g) and treated dropwise with aq. 5% (w/v) NH₄Cl (2.5ml) during 3min. After 5min the hot mixture was rapidly filtered into aq. 20% (w/v) NaCl (10ml) at –15°C. The precipitate that formed was quickly filtered off and washed with benzene at 10°C to give shiny colourless plates of N-hydroxy-[ethyl-¹⁴C]4-phenetidine (0.4g; 45% yield; specific radioactivity 33mCi/mol), m.p. 91°C (decomp.). The yield varied from 10 to 60% in different preparations. The compound decomposed on keeping in a dark bottle at –10°C for a few days. The filtrate and washing were evaporated *in vacuo*, the residue was extracted with ethanol (20ml) and the extract was evaporated *in vacuo*. Examination of the residue by t.l.c. (as above) revealed the presence of at least six products visible under u.v. light. The N-hydroxy derivative was soluble in ethanol, ether and ethyl acetate and insoluble in cold benzene, light petroleum and water. It decomposed within 2 days on storage in a dark bottle at 4°C, more rapidly in solution or in the presence of trace metals, impurities, heat, sunlight, acid or alkali, or during attempts to recrystallize it. The compound appeared generally much less stable than N-phenylhydroxylamine.

N-Acetoxy[ethyl-¹⁴C]phenacetin. A solution of N-hydroxy-[ethyl-¹⁴C]4-phenetidine (0.5g) in ether (100ml) containing acetic anhydride (2ml) was kept for 16h at 4°C and washed with ice-cold water (2×10ml), dried (over Na₂SO₄), evaporated *in vacuo* at 10°C and the residue recrystallized from acetone–light petroleum (b.p. 40–60°C) to give colourless plates of N-acetoxy[ethyl-¹⁴C]phenacetin (0.23g; specific radioactivity 33mCi/mol), m.p. 127°C (decomp.) (Found: C, 60.7; H, 6.4; N, 5.9; C₁₂H₁₅NO₄ requires C, 60.8; H, 6.4; N, 5.9%).

[diacetyl-³H]N-Acetoxyphenacetin. As described for the ethyl-¹⁴C-labelled analogue, a solution of N-hydroxy-4-phenetidine (1.53g) in ether (150ml) containing [³H]-acetic anhydride (2.2ml; 11.5mCi) gave [diacetyl-³H]N-acetoxyphenacetin (0.8g; specific radioactivity 0.5mCi/mmol), m.p. 127°C (decomp.).

RESULTS

Binding in vitro of radioactive label from labelled phenacetin and related compounds to DNA, RNA and bovine plasma albumin. Salmon sperm DNA (30mg), purified by a detergent–salt procedure (Kay, Simmons & Dounce, 1952) to a residual protein content of less than 0.2%, or highly polymerized yeast RNA (30mg) or bovine plasma albumin (30mg) was dissolved in 1mM-sodium citrate buffer, pH 7.4 (50ml). Each solution was treated with one of the following substances (0.25mmol; 8.25μCi): 4-nitro[ethyl-¹⁴C]phenetole, [ethyl-¹⁴C]4-phenetidine hydrochloride, [ethyl-¹⁴C]phenacetin, N-acetoxy[ethyl-¹⁴C]phenacetin, [acetyl-³H]phenacetin, [diacetyl-³H]N-acetoxyphenacetin, sodium [³H]acetate or [1-³H]ethanol. The last two were added as solutions in buffer (0.5ml) and the others as solutions

in acetone (0.5ml). Similar mixtures also contained resuspended washed rat liver microsomal fraction (equivalent to 2g of liver) or resuspended washed liver microsomal fraction from rats given intraperitoneal injections of 3-methylcholanthrene (25mg/kg body wt.) as a 5% (w/v) solution in arachis oil 40h previously, together with NADP⁺ (8mg), glucose 6-phosphate (38mg), magnesium chloride (15mg) and glucose 6-phosphate dehydrogenase (0.6 unit). The mixtures were shaken in air at 37°C for 1h. Those containing microsomal fraction were cooled in ice-water and the microsomal fraction was removed by ultracentrifugation at 80000g for 1h. The supernatant solutions and the remaining mixtures were extracted with chloroform (3 × 15ml), the aqueous fractions were treated with sodium chloride (2.5g) and the nucleic acid or protein was precipitated with ethanol (3 vol.), washed overnight in fresh solvent and dried in an evacuated desiccator (over phosphorus pentoxide). The DNA was deproteinized by a repetition of the detergent-salt procedure; RNA was further purified by the phenol method (Kirby, 1956) and protein by repeated precipitations with ethanol from solution in aq. 5% (w/v) sodium chloride until the final supernatant solution showed no residual radioactivity. Nucleic acids and proteins that had been treated with the *acetyl*-³H-labelled derivatives were finally washed with ethanol containing 0.1% (v/v) of acetic acid. DNA (2–5mg) was hydrolysed in deoxyribonuclease solution (0.5ml), RNA (2–5mg) was hydrolysed in ribonuclease solution (0.5ml) and protein (2–5mg) was dissolved in tetraethylammonium hydroxide solution (1.0ml) before the radioactivity was determined by liquid-scintillation counting. The results are shown in Table 1.

Binding in vivo of radioactive label to rat tissues after administration of radioactively labelled compounds. [*acetyl*-³H]Phenacetin (0.25mmol; 8.25μCi) was administered to each of two rats. Three other groups of two rats were similarly treated with one of the following compounds (0.25mmol; 8.25μCi): [*ethyl*-¹⁴C]phenacetin, [³H]ethanol and sodium [³H]acetate. The animals were killed after 16h, and acetone-dried powders of the various tissues listed in Table 2 were prepared and washed with cold ethanol until the final washing showed no residual radioactivity. DNA, RNA and protein were isolated from the liver by the method of Kirby (1956) as modified by Brookes & Lawley (1965), but the original method was used to remove polysaccharide. Acetone-dried powders, proteins and nucleic acids obtained from animals treated with [*acetyl*-³H]phenacetin or sodium [³H]acetate were washed with 0.1% (v/v) acetic acid in ethanol until the washings were free from radioactivity. Samples (5mg) of each acetone-dried powder and of the liver protein (5mg) were

Table 1. *Binding in vitro of radioactive label to protein and nucleic acids incubated with labelled phenacetin and related compounds or with [³H]ethanol or [³H]acetate*

Compound	$(10^{-6} \times \text{d.p.m.} / \text{g-atom of DNA P}^*)$			$(10^{-6} \times \text{d.p.m.} / \text{g-atom of RNA P}^*)$			$(10^{-6} \times \text{d.p.m.} / \text{mol of protein}^\dagger)$		
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
4-Nitro[<i>ethyl</i> - ¹⁴ C]phenetole	0	2.9	14.5	—	—	—	1.4	29	58
[<i>ethyl</i> - ¹⁴ C]4-Phenetidine	0; 1.5	15; 36	36; 59	—	—	—	5.9; 7.3	73; 94	422; 598
[<i>ethyl</i> - ¹⁴ C]Phenacetin	1.5; 0.7	87; 65	174; 144	0.7; 2.8	277; 365	423; 512	7.3; 14	420; 657	628; 792
N-Acetoxy[<i>ethyl</i> - ¹⁴ C]phenacetin	58; 73	116; 74	94; 58	141; 96	—	—	934	905	949
[<i>acetyl</i> - ³ H]Phenacetin	14; 10	51; 22	144; 175	58; 87	380; 438	642; 547	8760; 6424	3285; 2409	3066; 4552
[<i>diacetyl</i> - ³ H]N-Acetoxyphenacetin	949	—	—	3212	—	—	25549	20441	—
[³ H]Ethanol	0	1.5	1.5	0	73	88	0	58	81
Sodium [³ H]acetate	0	0	0	0	0	0	7.2	423	618

* Calculated on a basis of 8% P.

† Mol.wt. of bovine plasma albumin taken as 64000.

The isotopically labelled compound (0.25mmol; 8.25μCi) was incubated with 30mg samples of salmon sperm DNA, yeast RNA or bovine plasma albumin in (a) buffer, (b) buffer containing a microsomal hydroxylating system of rat liver or (c) buffer containing the same microsomal system from rats pretreated with 3-methylcholanthrene. Duplicate values, where shown, were obtained from repeat experiments. Other details are given in the text.

Extent of binding of radioactive label

Table 2. *Binding of radioactive label to rat tissues in vivo after administration of labelled compounds*

[*acetyl*- ^3H]Phenacetin (A), [*ethyl*- ^{14}C]phenacetin (B), sodium [^3H]acetate (C) or [^3H]ethanol (D) (each 0.25mmol; 8.25 μCi) was administered to starved rats. The radioactivity of acetone-dried powders of the tissues or of the nucleic acids and proteins isolated from the livers was determined after 16h. Other details are given in the text.

Powder or tissue fraction from	Extent of binding (d.p.m./mg) after administration of			
	(A)	(B)	(C)	(D)
Bladder	139	130; 156	14.6	7.1
Intestine	198	204; 438	29	14.2
Kidney	66	95; 73	12.8	13.3
Liver:				
Acetone-dried powder	81	88	22	28
Cytoplasmic protein	29	30	1.4	7.1
Nuclear protein	22	28	7.2	0.8
RNA	15	37	0	2.9
DNA	0.4	7.3	0	0.7
Lung	29	131; 234	2.2	37
Spleen	44	87; 117	0.7	7.2
Stomach	73	170; 249	29	15

dissolved in tetraethylammonium hydroxide solution (1.0ml); liver DNA (5mg) and RNA (5mg) were hydrolysed in deoxyribonuclease solution (0.5ml) and ribonuclease solution (0.5ml) respectively; the radioactivity of each solution was determined by liquid-scintillation counting. The results are shown in Table 2.

DISCUSSION

The carcinogenic aromatic amines and amides (e.g. 2-naphthylamine, 2-acetamidofluorene, 4-aminobiphenyl) and urethane, which produce tumours at sites remote from that of application, exert their carcinogenic activity after metabolic activation, and it appears likely that this metabolic activation to give 'proximate' carcinogens is a necessary prerequisite for the carcinogenesis and for the covalent binding of such compounds to cellular macromolecules (reviewed by Miller & Miller, 1966).

The present work (Table 1) shows that the ratios of the binding *in vitro* of radioactive label derived from [*ethyl*- ^{14}C]phenacetin, *N*-acetoxy[*ethyl*- ^{14}C]phenacetin, [*acetyl*- ^3H]phenacetin and [*diacetyl*- ^3H]N-acetoxyphenacetin per g-atom of DNA P, per g-atom of RNA P and per mol of protein in the absence of microsomal fraction are approx. 1:60:11:863, 1:68:41:1835 and 1:88:713:2399 respectively. The extent of binding of radioactivity derived from [*ethyl*- ^{14}C]phenacetin to nucleic acid or protein (Table 1) increases in the presence of the liver microsomal hydroxylating system and somewhat more so in the presence of microsomal fraction obtained from rats pretreated with 3-methylcholanthrene, which stimulates the metabolism of

phenacetin in rats and cats (Welch, Conney & Burns, 1966). [*acetyl*- ^3H]Phenacetin gave similar results with respect to binding to nucleic acids; compared with the *ethyl*- ^{14}C -labelled analogue this compound showed a higher degree of binding (Table 1) to protein in the absence of the microsomal hydroxylating system.

These results indicate that the binding of radioactive label derived from labelled phenacetin to nucleic acids and proteins, other than by protein acetylation, is facilitated by metabolic activation of phenacetin. This is further supported by the following: (a) radioactive label from labelled *N*-acetoxyphenacetin, which is a likely metabolite of phenacetin, binds more extensively than does radioactive label from labelled phenacetin to the nucleic acids and protein (Table 1) in the absence of a metabolizing system; (b) radioactive label from labelled 4-phenetidine and 4-nitrophenetole bind appreciably to DNA and protein in the presence, but not in the absence, of a metabolizing system. The relatively low extent of binding of radioactive label from labelled 4-nitrophenetole in the microsomal system may reflect the fact that the nitro reductase of mammalian liver microsomal fraction is inhibited by oxygen (Saz & Slie, 1954; Fouts & Brodie, 1957), which was not excluded during the incubations.

Radioactive label from labelled phenacetin is bound *in vivo* to all tissues examined (Table 2). The relatively high degree of binding to stomach and intestinal tissues may be related to the gastrointestinal disorders that, like acetanilide, phenacetin sometimes causes. The binding to kidney and bladder tissue may be related to the nephrotoxicity and possible carcinogenicity of the drug to

these organs (Zeman, 1963; Abrahams, Rubenstein, Levin & Wunderlich, 1964; Angervall *et al.* 1969).

The binding *in vivo* of radioactive label from [*acetyl*-³H]phenacetin may result partly from acetylation of tissue proteins. This is supported by the finding that the degree of binding of radioactive label from [*acetyl*-³H]phenacetin and [*diacetyl*-³H]-*N*-acetoxyphenacetin to bovine plasma albumin *in vitro* (see Table 1) and of the former to liver proteins *in vivo* (Table 2) is appreciably greater than that from the corresponding *ethyl*-¹⁴C-labelled analogues. The implications of this result are not clear, but the occurrence of *N*-acetyl groups in a number of proteins (Narita, 1958; Marshall & Neuberger, 1961; Phillips, 1963) and the acetylation of proteins by acetylsalicylate (Pinckard, Hawkins & Farr, 1968) have been described. Acetylation decreases the effectiveness of histones as inhibitors of RNA polymerase (Allfrey, Faulkner & Mirsky, 1964).

The greater binding of radioactive label from [*ethyl*-¹⁴C]phenacetin to liver nucleic acids as compared with that from [*acetyl*-³H]phenacetin (see Table 2) indicates that phenacetin, like many chemical carcinogens, may be an alkylating agent *in vivo*.

As phenacetin is metabolized mainly by de-ethylation and by deacetylation to a minor extent (Brodie & Axelrod, 1949; Smith & Williams, 1949) it is possible that the binding of radioactive label may be due to the incorporation of labelled C₂ units into the biological macromolecules by normal biosynthetic pathways. Since the ethyl group of phenacetin is known (Axelrod, 1956) to be metabolically converted into acetaldehyde, which is reversibly formed from ethanol in the presence of NAD⁺ and alcohol dehydrogenase (Westheimer, Fisher, Conn & Vennesland, 1951), and since both ethanol and acetaldehyde are further oxidized into acetate, the contribution of incorporation of C₂ units to the total binding might be determined by a comparison of the relative extents to which radioactive label derived from equimolar amounts of labelled ethanol, acetate and phenacetin is bound. Such a comparison (see Tables 1 and 2) reveals that the binding of radioactive label derived from labelled ethanol and acetate to nucleic acids and proteins *in vitro* (Table 1) and to body tissues, liver nucleic acids and liver proteins *in vivo* (Table 2) either does not occur or occurs to a significantly smaller extent than when derived from labelled phenacetin. Hence incorporation of labelled C₂ units can account for only a part of the total bound radioactive label. The finding that radioactive label from labelled acetate is incorporated into proteins but not into nucleic

acids either *in vivo* or *in vitro* agrees with previous reports (Phillips, 1963; Paik & Kim, 1970).

These results indicate that the binding of radioactive label derived from [*acetyl*-³H]phenacetin and [*ethyl*-¹⁴C]phenacetin described in this work may result from (a) the transfer of [*acetyl*-³H] groups to proteins *in vivo* and *in vitro*, (b) the entry of labelled C₂ units into the general metabolic pool and incorporation of radioactivity by normal metabolic pathways *in vivo* and (c) the binding of phenacetin or its metabolites *in vivo* or in the presence of a rat liver microsomal system *in vitro*.

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