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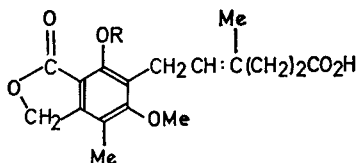
The metabolism and binding of [^{14}C]mycophenolic acid in the rat

R. NERY AND E. NICE

Chester Beatty Research Institute, Institute of Cancer Research, Fulham Road, London, S.W.3, U.K.

Seventeen h after the intraperitoneal administration of 33 mg/kg of [^{14}C]mycophenolic acid to rats, radioactivity was bound to the tissues of the intestines, bladder, stomach, kidney, liver and lung in decreasing order; no binding to spleen tissue was observed. *In vitro* incubations of the agent with macromolecules resulted in the binding of radioactivity to salmon sperm DNA and to bovine plasma albumin, the extent of binding being increased and decreased, respectively, in the presence of a rat liver microsomal system. The binding was apparently covalent since repeated purification procedures failed to release the bound radioactivity; heating of [^{14}C]mycophenolic acid bound-DNA in N hydrochloric acid at 100° for 2 h caused the release of the bound radioactivity. Under the conditions described, 43% of the administered radioactivity was excreted in the urine (33%) and faeces (10%); the urine contained free mycophenolic acid (13%), mycophenolic acid glucosiduronate (17%) and an uncharacterized metabolite (3%).

Mycophenolic acid (I) a metabolite of several strains of *Penicillium brevi-compactum* (Clutterbuck, Oxford & others, 1932) was first detected by Gosio (1896), isolated and named by Alsberg & Black (1913) and structurally characterized by Raistrick and his co-workers (Clutterbuck & Raistrick, 1933; Birkinshaw, Bracken & others, 1948; Birkinshaw, Raistrick & Ross, 1952). It is an antibiotic (Abraham, 1945; Gilliver, 1946; Brian, 1949) of low toxicity (Carter, Franklin & others, 1969) which also has antimitotic (Carter & others, 1969) and anti-cancer (Carter, 1966; Williams, Lively & others, 1968; Carter & others, 1969) properties; it is also a potent inhibitor of nucleic acid synthesis largely through inhibition of inosinic acid dehydrogenase (Franklin and Cook, 1969; Carter & others, 1969) which converts inosinic acid into xanthylic acid. These properties and possible clinical use of mycophenolic acid against human malignancies seemed to justify an investigation into the metabolism, distribution and tissue binding properties of this agent.



I Mycophenolic acid (R = H) and II mycophenolic acid glucosiduronate (R = $C_6H_9O_6$).

METHODS AND MATERIALS

Radioactive assays were performed on a Packard Tri-carb liquid scintillation spectrometer (Model 3375) and represent counts at least 50% above background corrected for quenching by addition of an internal standard.

Incubations, unless otherwise stated, were performed by shaking the mixtures in a

metabolic shaker (H. Mickle, Gomshall, Surrey) at 65 excursions/min in air at 37°.

Animals. Male 6 week old Chester Beatty rats (150 g) were used for the preparation of liver microsomes or were given intraperitoneal injections of [^{14}C]mycophenolic acid (sp. activity, 0·925 μCi/mg; dose, 30·8 μCi/kg) as a 2% (w/v) solution in polyethylene glycol. In metabolic experiments, the animals were starved for 8 h preceding treatment and for the duration of the experiment; the animals were housed in a polythene cage lined with two sheets of Whatman 3MM filter paper held in position with glass rods. Water was freely available.

Chromatography. Whatman 3MM chromatography paper was used for ascending overnight development in (a) methanol–6N HCl (7:3, by vol) and for descending development in (b) butan-1-ol–acetic acid–water (12:3:5, by vol) and (c) butan-1-ol–propan-1-ol–2M NH_3 (2:1:1, by vol). For t.l.c., glass plates were coated with silica gel G (E. Merck, Darmstadt, W. Germany) of 0·25 mm thickness and the chromatograms were developed in (d) benzene–ethanol (1:1). Mycophenolic acid on chromatograms was detected by ultraviolet light from a chromatolite lamp (Hanovia, Ltd.) and had R_F values 1·0, 0·96, 0·42 and 0·85 in solvents (a) to (d) respectively.

Materials

[^{14}C]Mycophenolic acid (sp. activity, 592 mCi/mol) and mycophenolic acid were kindly supplied by Drs. A. Hayes and T. J. Franklin of Imperial Chemical Industries Ltd., Aldersley Park, Macclesfield, Cheshire, U.K.; the radioactive material was diluted with the unlabelled material as required. DNA (from salmon testes) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; bovine plasma albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.; $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase (from yeast) were from Boehringer Corp. (London) Ltd., London, W.5, U.K.; sulphatase (Taka-diastase, from *Aspergillus oryzae*) was from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, U.K.; β-glucuronidase (Ketodase, from bovine liver) was from Warner-Chilcott, Morris Plains, N.J., U.S.A.; and deoxyribonuclease I (D) from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Washed rat liver microsomes were prepared as described by Booth & Boyland (1964).

RESULTS

Metabolism of [^{14}C]*mycophenolic acid*

[^{14}C]Mycophenolic acid (10 mg, 9·25 μCi) was administered to two rats and the urine and faeces were collected for 17 h. Radioactive assay revealed that the faeces contained approximately 10% of the administered radioactivity. The filter paper, glass rods and inside of the cage were washed with portions (200 ml) of aqueous 50% ethanol until no more radioactivity could be extracted. The washings were combined and evaporated in a vacuum and the residue was dissolved in water (10 ml). Radioactive assays of aliquots (0·5 ml) revealed that approximately 33% of the administered radioactivity was excreted in the urine. Chromatography of aliquots (0·1 ml) of the urine concentrate in solvent (c) revealed three areas of radioactivity centred at R_F 0·07, R_F 0·30 and R_F 0·42 representing 17, 3 and 13%, respectively, of the administered radioactivity (see Fig 1). Aliquots (1·0 ml) of the urine concentrate were diluted with 0·2M phosphate buffer, pH 7·0 (0·2 ml) and incubated for 24 h with or without β-glucuronidase ("Ketodase", 0·2 ml; 1000 Fishmann units) or sulphatase (Taka-diastase, 20 mg). Examination of the incubates by chromatography in solvent

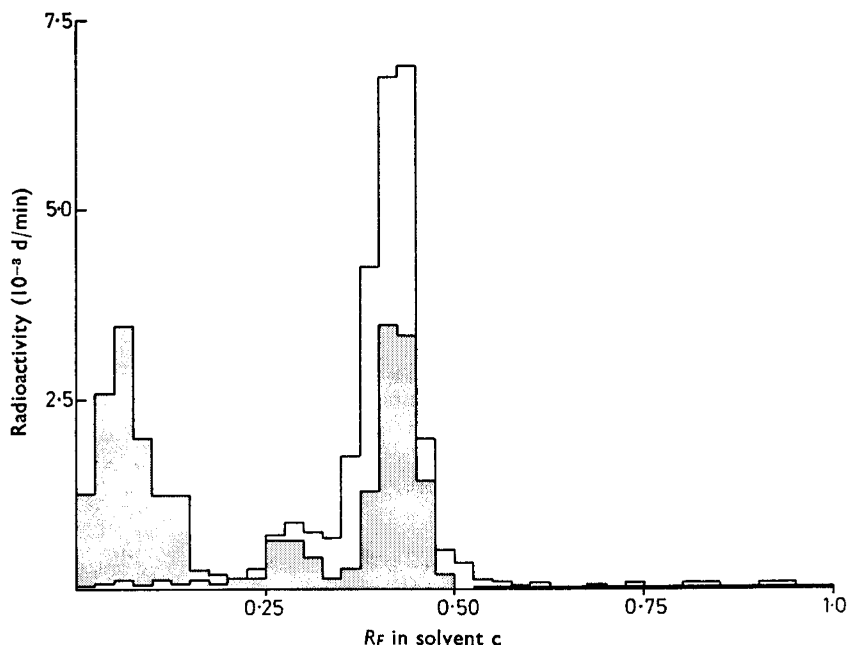


FIG. 1. Histogram of metabolites of [^{14}C]mycophenolic acid excreted in the urine (stippled areas) and of the same after incubation with β-glucuronidase (clear areas). Other details are given in the text.

(c) showed that the radioactivities of the three components in the β-glucuronidase-treated mixture were 1·3, 10·0 and 87·8 % respectively, of the applied radioactivity and were unchanged in the two other mixtures (see Fig. 1).

Aliquots (1 ml) of the urine concentrate, before and after hydrolysis with "Ketodase", were heated with mycophenolic acid (0·4 g) to give clear solutions which, on cooling, deposited colourless prisms, m.p. and mixed m.p. 141°, of mycophenolic acid which was recrystallized from water to constant activity. The results showed that the urine specimen before and after hydrolysis with the enzyme contained 13 and 30%, respectively, of the administered radioactivity as mycophenolic acid.

The binding of [^{14}C]*mycophenolic acid to rat tissues* in vivo

The rats used in the metabolic experiments described above were killed by cervical dislocation 17 h after administration of [^{14}C]mycophenolic acid. Acetone powders of tissues were prepared and washed repeatedly with acetone until the final washing entrained no radioactivity as determined by scintillation counting. Samples (2 mg) were dissolved in tetraethylammonium hydroxide (0·5 ml) and assayed for radioactivity. The results are shown in Table 1.

The binding of [^{14}C]*mycophenolic acid to salmon sperm DNA and to bovine plasma albumin* in vitro

Duplicate solutions of salmon sperm DNA (30 mg), purified by a detergent-salt procedure (Kay, Simmons & Dounce, 1952) to a residual protein content of less than

Table 1. *Radioactive binding of* [^{14}C]*mycophenolic acid to rat tissue* in vivo *and to DNA and protein* in vitro. [^{14}C]Mycophenolic acid was incubated at pH 7·4 for 1 h at 37° in air with the protein or DNA in the presence or absence of a rat liver microsomal system; or the acid was administered intraperitoneally to rats and acetone powders of the various tissues were prepared 17 h later. Further details are given in the text.

Binding site	Extent of binding*
A. *In vitro*	
Salmon sperm DNA	(i) 0·02, 0·03
	(ii) 0·77, 0·89
Bovine plasma albumin	(i) 2500, 1968
	(ii) 704, 642
B. *In vivo*	
Intestine	1·02
Bladder	0·32
Stomach	0·16
Kidney	0·05
Liver	0·04
Lung	0·02
Spleen	0

* μMol/g atom of DNA-P (assuming 8% P) or per mol of protein (assuming mol wt of 64 000) or per g of acetone powder of tissue. (i) In the absence and (ii) in the presence of rat liver microsomes.

0·2%, or bovine plasma albumin (30 mg) in 1 mM sodium citrate buffer, pH 7·4 (50 ml) was treated with a solution of [^{14}C]mycophenolic acid (0·2 mg, 0·37 μCi) in acetone (0·2 ml). Similar mixtures also contained resuspended washed rat liver microsomes (equivalent to 2 g liver), NADP$^+$ (8 mg), glucose 6-phosphate (38 mg), $MgCl_2$ (15 mg) and glucose 6-phosphate dehydrogenase (0·6 unit). The mixtures were incubated for 1 h; microsomes were removed from the microsome-containing mixtures by ultra-centrifugation at 80 000 *g* for 1 h. The supernatant solutions and the remaining mixtures were extracted with chloroform (3 $\times$ 2 ml), the aqueous fractions treated with NaCl (2·5 g) and the nucleic acid or protein was precipitated with ethanol (3 vol) or acetone (3 vol) respectively, washed overnight in fresh solvent and dried in an evacuated desiccator over P_2O_5. The DNA was further purified by a repetition of the detergent-salt procedure and the protein by repeated precipitations with acetone from solution in aqueous -5% (w/v) NaCl until the final supernatant contained no radioactivity. DNA (2 mg) was hydrolysed by incubation in deoxyribonuclease solution (0·5 ml; 7500 units) and protein (2 mg) was dissolved in tetraethylammonium hydroxide (0·5 ml). The radioactivities were determined by scintillation counting. The results are shown in Table 1.

A sample (2 mg, 2534 d/min) of the purified DNA obtained from the DNA-[^{14}C]-mycophenolic acid–microsome mixture was heated in N HCl (1·0 ml) at 100° for 2 h. Chromatography of the hydrolysate (0·1 ml) in solvents (a), (b) or (c) revealed (i) a major area of radioactivity (comprising approx. 93% of the radioactivity) at R_F 0 to R_F 0·1 in solvents (b) and (c) and at R_F 0·31 to R_F 0·54 centred at R_F 0·38 in solvent (a) and (ii) a minor area (comprising the remainder of the radioactivity) at R_F 0·95 to R_F 1·0 in solvents (a) and (b) and at R_F 0·40 to R_F 0·44 in solvent (c); (ii) appeared to be due to mycophenolic acid which showed similar R_F values in the three solvents.

Failure of [^{14}C]*mycophenolic acid to react* in vitro *with purines, pyrimidines and* N-*acetylcysteine*

Solutions of [^{14}C]mycophenolic acid (1 mg, 0·185 μCi) in acetone (0·1 ml) were severally treated with solutions of each of the following substances (2 mg) in 1 mM-citrate buffer, pH 7·0 (0·2 ml): guanine, adenine, cytosine, thymine, uracil and *N*-acetylcysteine. The mixtures were heated at 60° for 4 h and aliquots (0·1 ml) examined by chromatography in solvents (a), (b) and (c). Radioactive assay of strips (2 cm wide by 1 cm along the direction of development) showed that [^{14}C]mycophenolic acid was the only radioactive component of all the mixtures.

DISCUSSION

Administration of [^{14}C]mycophenolic acid into the intraperitoneal cavity of rats resulted in the uneven binding of radioactivity to all tissues examined except the spleen. The highest level of binding occurred in the tissues of the gastrointestinal tract (see Table 1); this result may be related to the observation that mycophenolic acid caused the development of abdominal colic, watery blood-stained diarrhoea and cytotoxic damage to the intestinal mucosa in rhesus monkeys (Carter & others, 1969). Radioactivity from [^{14}C]mycophenolic acid was strongly bound to rat tissues *in vivo* and to salmon sperm DNA and bovine plasma albumin *in vitro*. The binding to DNA was probably covalent since repeated purification procedures failed to release the bound radioactivity; heating of radioactivity bound-DNA at 100° for 2 h in N hydrochloric acid produced a small amount of a radioactive substance chromatographically identical with mycophenolic acid and an unidentified radioactive product having R_F 0·38 in solvent (a). The binding of radioactivity to DNA was apparently potentiated by metabolism since the extent of binding to salmon sperm DNA increased 30 to 37-fold on incubation in the presence of a rat liver microsomal system. The binding to bovine plasma albumin showed the reverse effect (see Table 1); the reason for this is not clear but may be due to competitive binding of radioactivity to the microsomes. The relatively higher binding to bladder than to kidney may indicate hydrolysis of mycophenolic acid β-glucosiduronate by the β-glucuronidase of urine; this indicates that at least part of the free mycophenolic acid found in the urine might have originated in this way and that the glucuronide is less strongly bound than the parent compound.

Mycophenolic acid was apparently not extensively metabolized in the rat. Approximately 43% of the administered radioactivity was excreted in the urine (33%) and faeces (10%) 17 h after administration of a dose of 33 mg/kg. The products excreted in the urine comprised mycophenolic acid (13%), mycophenolic acid glucosiduronate (17%) and a minor uncharacterized metabolite (3%). Fig. 1 shows that the glucuronide, which was probably the phenolic *O*-derivative (II), was almost quantitatively converted into mycophenolic acid by the action of a bacterial β-glucuronidase (Ketodase); this result was confirmed by isotope dilution analysis of the urine extract before and after the enzymic hydrolysis. None of the three urinary metabolites was changed by the action of a sulphatase (Taka-diastase).

Since the [^{14}C]mycophenolic acid used was biosynthesized from [1-^{14}C]acetate, alternate carbon atoms of the biosynthetic acid might have been radioactively labelled. Metabolic breakdown of the molecule might generate radioactive one-carbon or two-carbon precursors of tissue components; hence, part of the bound tissue radioactivity

might result from incorporation, by normal biosynthetic pathways, of such precursors arising from the methyl, methoxy or side-chain groups if these were radioactively labelled. Such incorporation is likely to occur *in vivo* into tissue proteins, among other components such as lipid and carbohydrate; this is unlikely to occur significantly *in vitro* in the absence of the microsomal system or to nucleic acids *in vivo* or *in vitro* since fragments arising from radioactively labelled ethanol or acetate are not actively incorporated into rat liver nucleic acids *in vivo* or *in vitro* (Paik & Kim, 1970; Nery, 1971).

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