30 mg/kg.[9] Reactivation to a new steady state in the region of 20–30 per cent of normal took 45–50 min. When concentrations of TMB-4 in the region of those found in the diaphragm *in vivo* after administration of therapeutic doses[10] were added, reactivation was so rapid that no transitional curve was seen between initial and final velocities.

Inhibition of rat preparations by the same concentrations of TEPP was extremely rapid, the transition curves being mostly too short for the estimation of rate constants. The magnitudes of the inhibitions were much the same as in the guinea pig. Addition of 0·1 mM-P2S gave extremely rapid reactivation, again to around 25 per cent.

These observations lead to the supposition that a major factor in the "protective" action of TEPP is the speed of the inhibition and reactivation processes. We found[1] that if TEPP were given to guinea pigs only 1 min before Soman the "protective" effect was little better than if only atropine and P2S were given, whereas the maximum "protection" was found if the interval between TEPP and Soman was 0·5–5 hr. This is evidently a reflexion of the relatively slow rate of inhibition of AChE by TEPP. P2S is an effective oxime in the guinea pig, possibly because the slow reactivation lags behind the clearance of Soman from the diaphragm, whereas it is ineffective in the rat because the rapid reactivation occurs while there is still enough free Soman present to re-inhibit the reactivated fraction of enzyme. Similarly rapid reactivation by TMB-4 explains its therapeutic ineffectiveness in the guinea pig when used in this manner. It is not yet possible to refine this hypothesis by studies of changes in the concentrations of free TEPP or Soman, because no method is known whereby the nanogram quantities of organophosphate can be estimated in tissues.

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Inhibition of guanine nucleotide biosynthesis by mycophenolic acid in Yoshida ascites cells

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Mycophenolic acid suppresses the growth of a wide range of transplantable tumours in rats and mice.[1,2] Studies on the mode of action of the compound indicate that its main inhibitory effect is against the biosynthesis of guanine nucleotides.[3] We found that mycophenolic acid is a very potent inhibitor of inosine monophosphate (IMP) dehydrogenase.[3] Since these mode of action studies involved the addition of mycophenolic acid to cells and enzyme preparations *in vitro* we thought it important to study the effects of orally administered mycophenolic acid on the fate of [^{14}C]hypoxan-

thine in tumour cells. For this purpose we have chosen the Yoshida ascites tumour whose growth *in vivo* is extremely sensitive to mycophenolic acid.

Mycophenolic acid is rapidly converted in rat, mouse and man to a glucuronide derivative in which the phenolic hydroxyl group is substituted by glucuronic acid.[4] The glucuronide is about 2000 times less active against IMP dehydrogenase *in vitro* than mycophenolic acid itself (T. J. Franklin and N. M. Beard, unpublished results). The relative abilities of various tumours to convert mycophenolic acid glucuronide back to the free acid have been implicated in the marked differences in the intrinsic sensitivities of tumours to mycophenolic acid.[4] Sensitive tumours have in general been found to have high β-glucuronidase activity although the ability of these tumours to convert mycophenolic acid glucuronide to the free acid was not reported.[4] We have therefore tested the ability of Yoshida ascites cells to carry out this conversion *in vitro* and also compared the effects of mycophenolic acid glucuronide with the free acid on the conversion of [^{14}C]hypoxanthine into nucleic acid guanine by Yoshida cells.

MATERIALS AND METHODS

Chemicals

[8-^{14}C]Hypoxanthine 60·1 μc/μmole was obtained from the Radiochemical Centre, Amersham, Bucks. Dr. R. H. Williams of the Lilly Research Laboratories kindly provided a sample of mycophenolic acid glucuronide. Mycophenolic acid (disodium salt) itself is a product of Imperial Chemical Industries Ltd.

Incubation conditions

(a) Uptake of [^{14}C]hypoxanthine: Yoshida ascites fluid was withdrawn from rats 6–7 days after transplantation of the tumours and either used undiluted or first diluted 10 times with Krebs–Ringer phosphate medium (Ca^{2+}-free) containing 5·5 mM glucose, 1 mM glutamine and 2·3 mM glycine. Approximately 8×10^7 cells were incubated with gentle shaking with 1 μc of [^{14}C]hypoxanthine (0·016 μmole) dissolved in phosphate-buffered saline, pH 7·0 for 30 min at 37°. The uptake of radioactivity into nucleic acid guanine and adenine nucleotides was determined as previously described and was found to be linear over the period of incubation.[3] When the effects of mycophenolic acid and its glucuronide on the uptake of radioactivity were studied, the cells were always preincubated with the compounds before the addition of [^{14}C]hypoxanthine.[3]

(b) Effect of Yoshida cell preparations on mycophenolic acid glucuronide. Ascites fluid either undiluted or first diluted 10 times with the Krebs–Ringer medium described above, was incubated with 100 μg of mycophenolic acid glucuronide in a total volume of 1 ml for 1 hr at 37° with gentle shaking. An equal volume of ice-cold 0·5 N perchloric acid was then added and the mixture stood for 15 min at 0°. The precipitate was removed by centrifugation and the supernatant fraction was extracted three times with 2-ml aliquots of diethyl ether. The combined ether fractions were evaporated to dryness and the residue then redissolved in 0·4 ml of ether. The whole of this ether solution was subjected to thin layer chromatography on silica plates using ethyl acetate–formic acid (95:5) as the developing solvent. Mycophenolic acid is extracted readily into ether and the free acid has an R_f of 0·73 in the above chromatographic system. The glucuronide, which does not extract into ether, remains at the origin.

In some experiments, the ascites fluid was centrifuged and the sedimented cells resuspended in a volume of 0·9% NaCl solution equal to the original volume of ascitic fluid. The cells were disrupted in an M.S.E. 100 W ultrasonic disintegrator operated at full power for 20 sec. The sonicate was then incubated with mycophenolic acid glucuronide (100 μg/ml) for 1 hr at 37° and precipitation, extraction and chromatography carried out as described above.

RESULTS

Effect of oral dosing of mycophenolic acid on the fate of [^{14}C]*hypoxanthine in Yoshida ascites tumour cells*

White rats of the Alderley Park strain (150 g) were dosed orally with 30 mg of the disodium salt of mycophenolic acid/kg body weight (this dose repeated daily completely suppresses the growth of the Yoshida tumour) on the 6th day after inoculation with the Yoshida ascites tumour. The animals were killed at intervals up to 4 hr after dosing and the ascites fluid collected in a heparinized glass vessel and then immediately incubated with [^{14}C]hypoxanthine as described in Materials and Methods. Considerable variations were found in the total amounts of radioactivity incorporated into the nucleic

acids of tumour cells from several undosed control animals. However, the relative amounts of radioactivity found in the nucleic acid adenine and guanine nucleotides was remarkably constant in the tumour cells from the control rats. The effect of mycophenolic acid has therefore been represented as the amount of radioactivity incorporated into nucleic acid guanine expressed as a percentage of the total amount of radioactivity incorporated into nucleic acid adenine and guanine nucleotides. Figure 1 shows a fall in this value 30 min after dosing with mycophenolic acid; the maximum effect was observed 60 min after dosing. Over the next 4 hr the percentage radioactivity incorporated into guanine progressively increased. Maximum inhibition of the conversion of hypoxanthine to nucleic acid guanine appeared to lag about 30 min behind the peak concentration of mycophenolic acid found in the blood after an oral dose of 30 mg/kg (Franklin and Cook unpublished work; D. S. Platt personal communication).

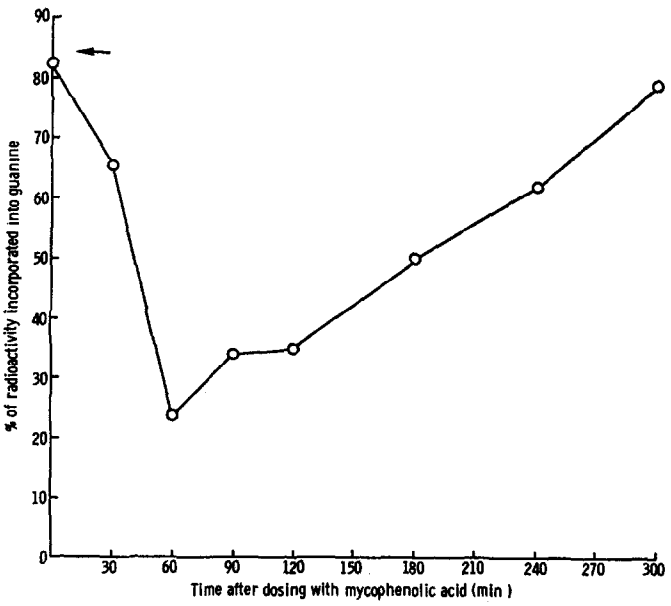

FIG. 1. Effect of an oral dose of mycophenolic acid (30 mg/kg) to rats bearing the Yoshida ascites tumour on the uptake of [^{14}C]hypoxanthine into nucleic acid guanine by the tumour cells *in vitro*. Samples of ascites fluid were removed at intervals after dosing and incubated for 30 min with [^{14}C] hypoxanthine. The incorporation of radioactivity into nucleic acid guanine is expressed as a percentage of the total radioactivity incorporated into nucleic acid adenine and guanine; the arrow indicates the percentage incorporated by tumour cells from undosed rats.

Effect of mycophenolic acid and its glucuronide on the incorporation of [^{14}C]*hypoxanthine into the nucleic acids of Yoshida tumour cells* in vitro

Mycophenolic acid had little effect on the percentage radioactivity incorporated into nucleic acid guanine at concentrations below 25 μg/ml when added to undiluted ascites fluid (Fig. 2). However, when mycophenolic acid was added to ascites fluid diluted ten times with Krebs–Ringer medium (Experimental Section) the compound then markedly depressed the uptake of radioactivity into nucleic acid guanine at 1 μg/ml. The glucuronide, in contrast had no effect at concentrations up to 50 μg/ml in either system (Fig. 2).

Effects of Yoshida tumour cell preparations on mycophenolic acid glucuronide

When the glucuronide of mycophenolic acid was incubated with Yoshida ascites fluid either undiluted or diluted ten times with Krebs–Ringer medium, there was no detectable release of free mycophenolic acid during the 1 hr period of incubation. Approximately 1 μg of mycophenolic can be detected on silica thin layer plates and the recovery of mycophenolic acid from acid aqueous solution into ether is not less than 95 per cent. Any conversion of the glucuronide to mycophenolic acid which may have occurred under the conditions of this experiment, cannot therefore have been more than 1 per cent of the total glucuronide present.

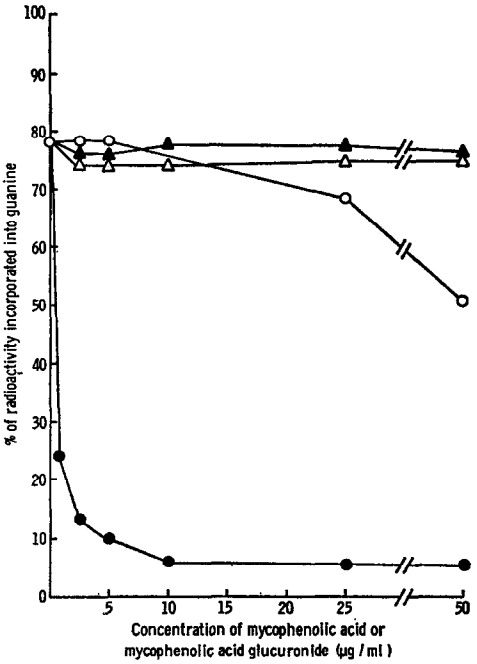

FIG. 2. Incorporation of radioactivity into nucleic acid guanine by Yoshida ascites tumour cells *in vitro* in the presence of mycophenolic acid or mycophenolic acid glucuronide; ○, △: mycophenolic acid and mycophenolic acid glucuronide respectively, added to undiluted ascites fluid, ●, ▲: mycophenolic acid and mycophenolic acid glucuronide respectively added to ascites fluid diluted 10 times with a Krebs–Ringer phosphate medium. The cells were preincubated with the drug or drug conjugate for 30 min before the addition of [^{14}C]hypoxanthine for a further 30 min. The incorporation of radioactivity into nucleic acid guanine is expressed as a percentage of the total radioactivity incorporated into nucleic acid guanine and adenine.

The "cryptic" β-glucuronidase activity of Yoshida ascites cells was readily demonstrated by prio disruption of the cells before incubation with mycophenolic acid glucuronide. Under these conditions the release of material having the same R_f as mycophenolic acid was easily detected. Disrupted tumour cells also readily released phenolphthalein from the corresponding glucuronide in 1 hr whereas the intact cells failed to carry out this conversion during the same period.

DISCUSSION

We have shown that oral administration of a therapeutic level of mycophenolic acid to rats bearing the Yoshida ascites tumour results in a striking inhibition of the incorporation of labelled hypoxanthine into nucleic acid guanine by the tumour cells *in vitro*. This is in accordance with the known ability of mycophenolic acid to inhibit the biosynthesis of guanine nucleotides when the antibiotic is added directly to various cell types *in vitro*.[3]

Since the maximum concentration of mycophenolic acid in rat blood and ascitic fluid following a single oral dose of 30 mg/kg body weight does not exceed 10 μg/ml (Franklin and Cook, unpublished observations and D. S. Platt, personal communication), it was surprising to find that addition of up to 25 μg of mycophenolic acid/ml to undiluted ascites fluid from control rats had no effect on the incorporation of [^{14}C]hypoxanthine into nucleic acid guanine. In view of the suggestion that the glucuronide of mycophenolic acid, which is rapidly formed *in vivo*, may be reconverted to the free acid by sensitive tumour cells[4] it seemed possible that this conversion could have resulted in substantially higher levels of intracellular mycophenolic acid following oral dosing. However, this hypothesis is weakened by the evident failure of mycophenolic acid glucuronide to inhibit the incorporation of radioactivity into nucleic acid guanine *in vitro* under conditions in which the free acid exerts very strong inhibition. The inactivity of mycophenolic acid glucuronide against the Yoshida tumour cells *in vitro* is readily explained by the observed inability of the intact cells to convert the

glucuronide to the free acid. Since β-glucuronidase is generally considered to be localized in the lysosomes, presumably the enzyme is normally inaccessible to its substrates in intact cells. Only when the lysosomes are disrupted, e.g. when the cells are lysed by ultrasound, can the glucuronides be attacked by β-glucuronidase. We therefore consider it unlikely that the β-glucuronidase activity of the Yoshida tumour cells can be a significant factor in determining the response of this tumour *in vivo* following administration of mycophenolic acid.

The failure of mycophenolic acid in concentrations as high as five times the maximum blood level to inhibit the conversion of hypoxanthine to guanine nucleotides by undiluted ascites fluid *in vitro* may be due to extensive serum protein binding of mycophenolic acid which reduces the concentration of the free antibiotic. This was first suggested by the observation that prior dilution of the ascites fluid resulted in a very striking increase in the activity of mycophenolic acid. Subsequently we have found by equilibrium dialysis studies that mycophenolic acid is indeed extensively bound by serum albumin (Franklin and Cook, unpublished work).

We are therefore left with the apparent discrepancy between the significant inhibition of the incorporation of radioactivity into nucleic acid guanine in undiluted ascites fluid *in vitro* which follows an oral dose of mycophenolic acid and the failure of the antibiotic, at a high concentration, to inhibit the same reaction when added directly to undiluted ascites fluid *in vitro*. It is conceivable that mycophenolic acid may be converted *in vivo* to some derivative either intrinsically more active than the parent compound or alternatively less extensively protein bound than mycophenolic acid. However, we have no evidence as yet for metabolic conversions of mycophenolic acid in the rat other than to the glucuronide.

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