INHIBITION BY CHLORAMPHENICOL OF THE MICROSOMAL MONOOXYGENASE COMPLEX OF RAT LIVER

P. E. B. REILLY and D. E. IVEY
The Department of Biochemistry, University of Queensland, St. Lucia, Qld., 4067, Australia

Received 17 October 1978

1. Introduction

In several species of animal the concomitant administration of chloramphenicol with a number of other therapeutic agents has been shown to prolong the actions of these agents [1–4]. Consistent with these observations was the finding that chloramphenicol could inhibit the drug metabolizing activity of a 9000 × *g* supernatant of an homogenate of liver tissue [5], and the antibiotic was further shown to be a competitive inhibitor of liver microsomal monooxygenase activity [6]. More recently however there have been reports [7–9] which have indicated that incubation of chloramphenicol with an oxygenated preparation of liver microsomes containing a system for generating reduced NADP results in the production of a metabolite of the antibiotic that binds covalently to microsomal protein. These findings would be consistent with the antibiotic being able to bring about irreversible inhibition of microsomal monooxygenase activity and they suggested to us the need to assess the claim, made in [1] where the experimental results were not given, that the antibiotic could cause irreversible inhibition in vitro of the monooxygenase activity of a 9000 × *g* supernatant fraction of an homogenate of liver tissue. However, since such a microsomal preparation contains unknown, and possibly variable, concentrations of endogenous substrates of monooxygenase activity, together with a variety of systems for generating reduced pyridine nucleotides, we selected as our enzyme preparation for the present studies resuspended washed liver microsomes.

In initial rate studies using this preparation we showed that chloramphenicol does both competitively and irreversibly inhibit monooxygenase activity with a half-life for inactivation of 11.5 min. We further showed that *o*-nitroanisole, a substrate for monooxygenase activity, profoundly inhibits the specific binding of radiolabeled material to microsomes incubated with ^{14}C-labeled chloramphenicol, and we consider that these collected results are sufficient evidence to warrant an investigation into the possible involvement of an active site-directed mechanism [10] in the inhibitory action of the antibiotic on microsomal monooxygenase activity.

2. Methods and results

Batches of microsomes were prepared at ~17 mg protein/ml by the procedure in [11] from liver tissue taken, on each occasion, from 2–4 sexually mature male Wistar rats, protein being determined on each batch by the Folin–phenol method [12]. The animals were all obtained from the University Central Animal Breeding Facility, and fed a stock laboratory diet until the day prior to sacrifice by decapitation. The monooxygenase activities of these microsomal preparations were measured at 37°C by a modification of a spectrophotometric method [11] in which O-demethylation of *o*-nitroanisole was measured. This method was chosen because:

(i) In principle it allows initial rates of enzymic activity to be measured directly, this being due to the development in the assay system of an absorption band associated with the production of *o*-nitrophenol;

(ii) *o*-Nitroanisole is a substrate for only one oxidation reaction, its use thereby obviating the complexities of interpreting reaction kinetics where this is not the case [13].

To realise the full potential of the method however it was modified in two ways

(1) Nicotinamide was omitted from the reaction mixture, thereby obviating the inhibitory effect of this compound on microsomal monooxygenase activity [14,15]

(2) NADP was included in the blank as well as in the sample cuvette.

This latter modification was found to be necessary for the accurate determination of initial rates of O-demethylation of *o*-nitroanisole owing to the development of an absorption band with a peak at 425 nm, which resulted from the action of reduced NADP on the microsomal preparation; and which was not taken into account in the blank cuvette in the original method. All the spectrophotometric assays were made by recording the linear increases in A_{430}, for a period of 5–10 min after initiation of the O-demethylation reaction, using a Varian Superscan 3, twin-beam, recording spectrophotometer set at 1.0 nm spectral band width and full scale deflection of 0.1 *A* units.

The method was shown to give a linear increase in reaction rate for increases in protein concentration in the assay over 0.25–1.0 mg protein/ml and fig 1 shows the competitive nature of the inhibition of microsomal activity resulting from inclusion of chloramphenicol at 1.0 mM in the reaction conditions detailed in the legend to fig 1. This result is in agreement with data obtained in [6] but may be considered more compelling since initial rates were measured in the absence of an inhibitor of monooxygenase activity.

Having obtained confirmation of the competitive nature of the inhibition of microsomal monooxygenase activity by chloramphenicol we next determined the effect of incubating oxygenated microsomes at 37°C with reduced NADP in the absence and presence of 1.0 mM inhibitor for progressively increasing periods prior to starting the spectrophotometric assay for monooxygenase activity by the addition of substrate. The details of the procedures are given in the legend to fig.2 which shows the results of this experiment, increasing the duration of preincubation of microsomes

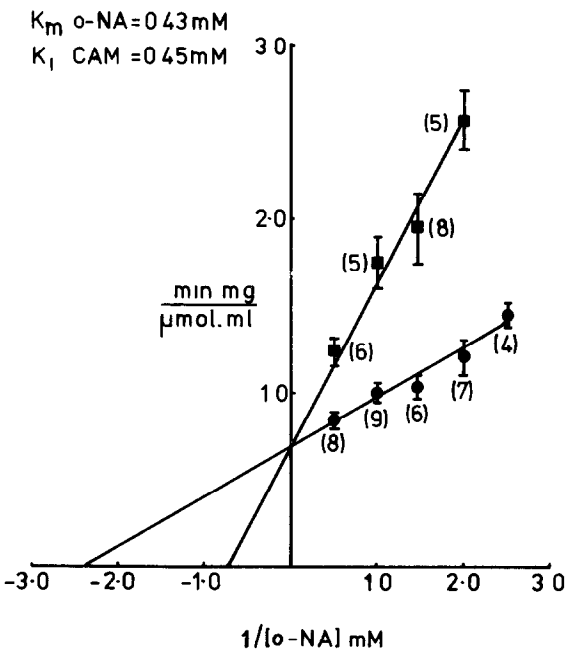

Fig.1 Chloramphenicol inhibition of microsomal O-demethylation of *o*-nitroanisole The assays were carried out at 37°C in total vol. 3 7 ml in 1 cm path length, stoppered, matched, silica cuvettes, both blank and sample reaction mixtures containing M/15 sodium phosphate buffer (pH 7.9), 2 5 mM glucose-6-phosphate, 50 μM NADP and 1 μl glucose-6-phosphate NADP oxidoreductase suspension (EC 1 1 1 49, 140 units/mg protein, 1 mg protein/ml). The sample cuvettes contained different aliquots of an 8 22 mM solution of *o*-nitroanisole in the phosphate buffer and the reactions were started in all cases by the addition of an aliquot of microsomal suspension first to the blank and then to the sample cuvette, to give 1 0 mg microsomal protein/ml In those assays where the effect of 1 0 mM chloramphenicol was being determined the antibiotic was dissolved in a solution of glucose-6-phosphate made up in the phosphate buffer and aliquots of this solution were dispensed into both the blank and sample cuvettes to give the required final concentrations of these constituents in the reaction mixtures All the solutions of reagents were prepared freshly for use each day and with the exception of the solutions of NADP were maintained at room temperature and bubbled with oxygen throughout the period in which the assays were being performed. The rates of change of absorbance were converted to rates of change of molarity of *o*-nitrophenol by dividing by 3550, this having been found to be the molar absorptivity of this product of the reaction under these conditions The means and standard errors of the means of the reciprocals of the activities of microsomes in the absence (●——●) and presence (■——■) of chloramphenicol are shown with the numbers of determinations given in parentheses beside each datum point

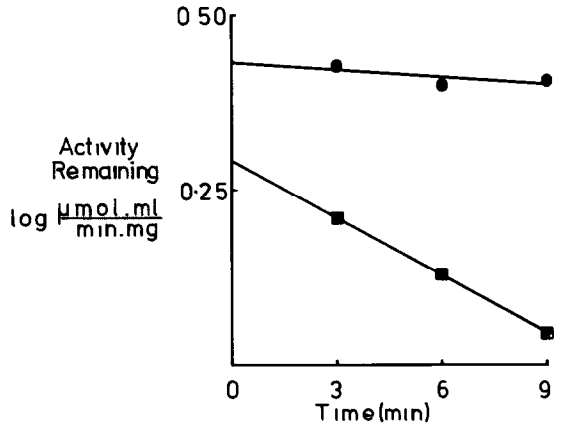

Fig.2 The effect of preincubation of microsomes with chloramphenicol Microsomes at 1 5 mg protein/ml were incubated aerobically at 37°C for 3, 6 or 9 min with or without 1 0 mM chloramphenicol in the presence of 50 μM NADP, 2 5 mM glucose-6-phosphate, 2 μl glucose-6-phosphate NADP oxidoreductase suspension and M/15 sodium phosphate buffer (pH 7 9) in total vol 6 0 ml At the end of each of the preincubation periods 2 4 ml aliquots of the mixture were dispensed into blank and sample spectrophotometer cuvettes and to each was immediately added 0 38 ml of a solution of 500 μM NADP, and 25 mM glucose-6-phosphate in M/15 sodium phosphate buffer (pH 7 9) containing 1 μl glucose-6-phosphate NADP oxidoreductase, which had been preincubated at 37°C for 3 min. The spectrophotometric assay of O-demethylase activity was started immediately after this addition of the reduced NADP-generating system by dispensing 0 9 ml of an 8.22 mM oxygenated solution of *o*-nitroanisole in the same buffer to the sample cell, linear increases in A_{430} being again recorded for about 5–10 min The rates of change in absorbance were converted to changes in molarity of *o*-nitrophenol as described in the legend to fig 1 and the results expressed as log of activity remaining plotted against the duration of preincubation of microsomes without (●——●) and with (■——■) 1 0 mM chloramphenicol, each datum point being the mean of two determinations The $t_{½}$ for inactivation of microsomal monooxygenase activity by chloramphenicol is 11.5 min

with chloramphenicol clearly resulting in an exponential loss of monooxygenase activity. These results are consistent with the concept of the enzyme becoming irreversibly inhibited and direct tests were carried out to confirm the possibility that chloramphenicol could in fact cause irreversible inhibition of monooxygenase activity.

To do this a comparison was first made between the catalytic activity of a microsomal preparation made from liver tissue taken from rats 1 h after they had received an intraperitoneal injection of chloramphenicol sodium succinate at a dose rate of 100 mg chloramphenicol/kg body wt, and that of a preparation made from liver tissue of rats which had received an injection of non-pyrogenic physiological saline. All the assays were carried out at 1.0 mg microsomal protein/ml and at 2.0 mM substrate under the conditions described for control microsomes in the legend to fig.1. This concentration of *o*-nitroanisole was selected because it lies in the region approaching zero order kinetics for the control microsomal preparations. The mean activity for the preparation made from the saline-treated rats was 3.26 ± 0.11 (9) $\mu mol.min^{-1}$ .mg $protein^{-1}$ ml^{-1} while that for the preparation made from the animals which had been pretreated with the antibiotic was only 1.92 ± 0.09 (8) $\mu mol.min^{-1}$ .mg $protein^{-1}$ $.ml^{-1}$. The difference between these mean values was significant by the grouped data student *t* test ($0.01 > P > 0.002$).

The second test was to incubate microsomes aerobically at 37°C with the system for generating reduced NADP described in the legend to fig.3, with and without 1.0 mM chloramphenicol for 6 min and then to submit 1 ml aliquots of these incubation mixtures to gel filtration at room temperature using 10 ml bed volume prepacked columns of Sephadex G-25 M (PD-10, Pharmacia), equilibrated with M/15 sodium phosphate buffer (pH 7 9). Fractions of 1 ml were collected, and protein freed from unbound antibiotic was recovered in fractions 3 and 4. The elution of free antibiotic did not start before fraction 6 with the peak concentration occurring in fraction 11, the antibiotic clearly exhibiting the characteristic retention of aromatic compounds on Sephadex G-25. After mixing the 2 protein-containing fractions to produce an homogeneous suspension aliquots were taken and assays of microsomal O-demethylase activity were carried out as described in the legend to fig.3. Figure 3 shows that the microsomes preincubated in the absence of chloramphenicol retained normal catalytic activity whereas those preincubated with the antibiotic showed kinetics which differed from these controls in 2 particular respects.

(1) The activities at all concentrations of *o*-nitroanisole were significantly lower than the corresponding activities of the control preparation, which shows that the separation of protein from

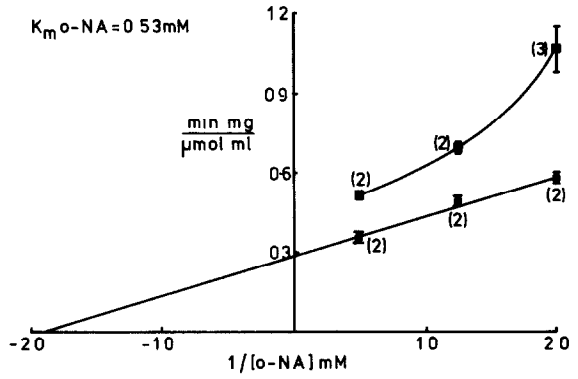

Fig.3. Catalytic activity of microsomes subsequent to a 6 min preincubation with or without chloramphenicol, followed by gel filtration Microsomes at 4.0 mg protein/ml were incubated aerobically at 37°C for 6 min with or without 1 0 mM chloramphenicol in the presence of 50 μM NADP, 2.5 mM glucose-6-phosphate, 1 μl glucose-6-phosphate NADP oxidoreductase, and M/15 sodium phosphate buffer (pH 7 9) in total vol 2 5 ml. At the end of this preincubation period two 1 ml aliquots were subjected separately to gel filtration, and the protein from each column was recovered quantitatively in single 2.0 ml fractions. These were then mixed to give a uniform suspension and 1.8 ml aliquots were immediately dispensed into blank and sample spectrophotometer cuvettes To each cuvette was immediately added 0.9 ml aliquot of a solution of 200 μM NADP and 10 mM glucose-6-phosphate, containing 2 μl glucose-6-phosphate NADP oxidoreductase, in M/15 sodium phsophate buffer (pH 7 9) which had been incubated at 37°C for 3 min The spectrophotometric assay of O-demethylase activity was initiated immediately thereafter by the addition of 1 0 ml oxygenated buffer to the blank cuvette and of different aliquots of an oxygenated 8 22 mM solution of *o*-nitroanisole in buffer to the sample cell, the volume being made to 1 0 ml by the further addition of oxygenated buffer solution. In this way all the assays were carried out at 1.0 mg microsomal protein/ml and where the substrate concentration varied between 0 5 mM and 2 0 mM The figure shows the means and standard errors of the means of the reciprocals of the activities of microsomes preincubated without chloramphenicol (•——•) and with the antibiotic (▪——▪) the numbers of observations being given in parentheses beside each datum point.

unbound antibiotic did not result in reversal of inhibition. This corroborated the indications from the previous 2 experiments that microsomal monooxygenase activity can be irreversibly inhibited by the antibiotic.

(2) The inhibited enzyme did not show Michaelis kinetics, the nature of the inhibition caused by the antibiotic not being identifiable directly from the double reciprocal plot. Figure 4 shows that this same result was obtained using microsomes prepared from livers of rats pretreated with chloramphenicol sodium succinate by intraperitoneal injection, the details of this experiment being given in the legend to this figure. Our interpretation of these two latter sets of data, both derived from experiments in which irreversibly-inhibited enzyme freed initially from unbound antibiotic was being used, was that under these assay conditions a degree of regeneration of active enzyme was occurring in a manner which showed some dependence on increasing substrate concentration.

Because chloramphenicol can cause both com-

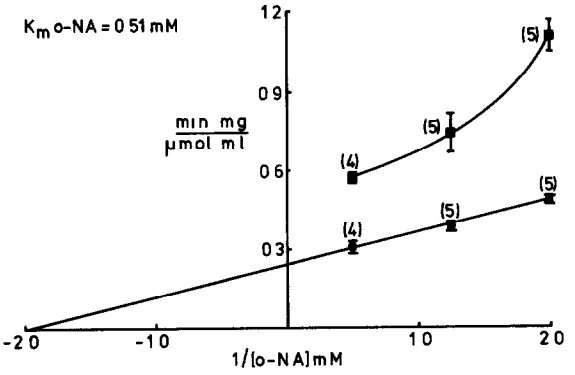

Fig.4 Catalytic activity of microsomes inhibited in vivo by intraperitoneal administration of chloramphenicol sodium succinate to rats. Two groups of 4 sexually mature male Wistar rats were used, each rat in one group receiving an intraperitoneal injection of sterile non-pyrogenic physiological saline, and each in the other group receiving an intraperitoneal injection of a solution of chloramphenicol sodium succinate dissolved in the saline solution at a dose rate of 100 mg chloramphenicol/kg body wt After 1 h each rat was sacrificed by decapitation and microsomes were prepared from pooled homogenates made from the livers of the animals in each group. The assays of O-demethylase activity of both preparations were carried out as described for the control microsomes in the legend to fig.1 and rates of production of *o*-nitrophenol were calculated as described in the legend to fig 1 The means and standard errors of the means of the reciprocals of the activities are shown for the microsomal preparations made from the livers of the saline-treated (•——•) and chloramphenicol-treated animals (▪——▪) the numbers of observations being given in parentheses beside each datum point.

petitive and irreversible inhibition of microsomal monooxygenase activity we considered that an active site directed mechanism may be involved in its action, and the following experiment, using dichloro [1-^{14}C]-acetyl chloramphenicol (Radiochemical Centre, Amersham, CFA515, batch 6, spec. radioact 7.18 mCi/mmol), was carried out to enable to preliminary assessment of this possibility to be made. Microsomes at 3.6 mg protein/ml were incubated aerobically in the presence of M/15 sodium phosphate buffer (pH 7.9) for 6 min in the presence of 0.1 mM radiolabeled chloramphenicol, control incubations being carried out in the absence of the NADPH generating system described in the legend to fig.1, and in the presence or absence of 2.0 mM *o*-nitroanisole. The total volume of all incubations was 1.25 ml. From each incubation mixture a 1 ml aliquot was subjected to gel filtration as described previously, the protein freed from unbound antibiotic being recovered quantitatively in a single 2 ml aliquot which was well mixed to give a homogeneous suspension. From this three 0.5 ml aliquots were taken into liquid scintillation vials and mixed with 5 ml of Riafluor (New England Nuclear). The resulting clear, homogeneous solutions were assayed for radioactivity using a Packard C_{2452} liquid scintillation spectrometer. No variation in quenching was evident between samples and all were counted at an efficiency for ^{14}C of 75%. The procedures to test for protection by *o*-nitroanisole against specific binding of radiolabeled material to microsomes were identical except that the NADPH generating system described above was included in the microsomal incubation step. After subtraction of the appropriate mean value for non-specific binding of radiolabeled material to microsomes obtained in the control experiments (expressed as pmol chloramphenicol.mg protein^{-1}) from the individual values obtained in each of the two test procedures estimates of radiolabeled material remaining specifically bound to microsomes were obtained. In the absence of *o*-nitroanisole the mean value for specific binding was 88.0 ± 17.1 (4) pmol.mg protein^{-1}, a value which is very close to that obtained in [7] for a control preparation of rat liver microsomes. The value obtained in the presence of 2.0 mM *o*-nitroanisole on the other hand was only 19.6 ± 6.6 (4) pmol.mg protein^{-1}, and the difference between these two mean values was significant by the grouped data student *t* test ($0.02 > P > 0.01$). This clearly demonstrates the protective effect against specific binding afforded by *o*-nitroanisole, and strongly suggests that an active site directed mechanism may be involved in the inhibition by chloramphenicol of microsomal monooxygenase activity.

3. Discussion and conclusions

Figure 1 clearly demonstrates that the determination of initial rates of microsomal monooxygenase activity can be satisfactorily carried out in the absence of nicotinamide and that chloramphenicol competitively inhibits O-demethylation of *o*-nitroanisole. The data in fig.2, on the other hand, show that microsomal monooxygenase activity can be progressively inhibited by increasing the duration of preincubation of microsomes with chloramphenicol in the presence of oxygen and a system for generating reduced NADP. This finding suggests that under these conditions chloramphenicol may cause irreversible inhibition of microsomal monooxygenase activity. This was confirmed by the data given in fig 3, the monooxygenase activities of microsomes preincubated with chloramphenicol, and separated from free antibiotic by gel filtration, being significantly less than those of control preparations at all substrate concentrations tested. Additional data confirming this action of the antibiotic are given in fig 4 where microsomes prepared from chloramphenicol pretreated rats, and used at a protein concentration equal to that used in the control experiments, are shown to be less catalytically active than those obtained from control animals which had received injections of saline only. These collected results, together with those from the experiment in which substrate was shown to protect against the specific binding of radiolabeled material to microsomes incubated with ^{14}C-labeled chloramphenicol, indicate the need to investigate the possibility that the antibiotic causes irreversible inhibition of microsomal monooxygenase activity by an active site directed mechanism. Such work is currently in progress. Further studies on reversal of inhibition, suggested to occur by the results shown in figs.3 and 4, are also being conducted.

References

[1] Dixon, R L and Fouts, J. R (1962) Biochem. Pharmacol. 11, 715–720

[2] Adams, H R. (1970) J Am. Vet Med Ass. 157, 1908–1913.

[3] Teske, R. H and Carter, G G (1971) J Am Vet Met Ass 159, 777–780

[4] Christensen, L K and Skovsted, L. (1969) The Lancet 2, 1379

[5] Mitoma, C , Yasuda, D , Tagg, J S., Neubauer, S E , Calderoni, F. J and Tanabe, M (1968) Biochem Pharmacol 17, 1377–1383.

[6] Grogan, D. E., Lane, M , Smith, F E., Bresnick, E. and Stone, K (1972) Biochem. Pharmacol 21, 3131–3144

[7] Krishna, G (1974) Postgrad Med J 50, suppl 5, 73–77

[8] Pohl, L R and Krishna, G (1978) Biochem. Pharmacol 27, 335–341

[9] Pohl, L R., Nelson, S. D and Krishna, G (1978) Biochem. Pharmacol 27, 491–496

[10] Baker, B R. (1967) Design of Active Site Directed Irreversible Enzyme Inhibitors, John Wiley and Sons, New York

[11] Netter, K J. (1960) Naunyn-Schmiedeberg's Arch. exp Path. u Pharmak 238, 292–300.

[12] Lowry, O. H , Roseborough, N J , Farr, A L and Randall, R J (1951) J Biol Chem. 193, 265–275.

[13] Gillette, J , Sasame, H and Stripp, B (1973) in Drug Metabolism and Disposition (Leibman, K C ed) vol 1, pp 164–175

[14] Schenkman, J B , Ball, J A and Estabrook, R W (1967) Biochem. Pharmacol 16, 1071–1081

[15] Sasame, H A and Gillette, J R. (1970) Biochem Pharmacol 19, 1025–1041.