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MICROSOMAL ENZYME ACTIVITY IN PERFUSED RAT LIVER

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Abstract—The activities of microsomal enzymes were observed during perfusion of livers isolated from both normal and phenobarbital treated rats. The hydroxylation of aniline and the *O*-demethylation of *p*-nitroanisol did not decrease substantially (by 10 per cent only) in the 9000 *g* fraction prepared from livers perfused for 4 hr.

N-demethylation of aminopyrine was reduced by 60 per cent in the same experimental conditions. A similar decrease of *N*-demethylation was also observed when livers were isolated from animals treated with phenobarbital. The content of cytochrome P-450 in the microsomes was stable during a 4-hr perfusion of isolated liver. The decrease of *N*-demethylation of aminopyrine could be partly restored by the simultaneous infusion of nicotinic acid, which is a precursor of NAD and NADP biosynthesis.

INTRODUCTION

THE PERFUSION of isolated liver is widely used for studies on drug metabolism. There are three main advantages of this experimental technique:[1]

(1) Liver is considered the most important site of metabolism for the majority of drugs. It is therefore very useful to evaluate its own contribution to the overall drug metabolism without the interference of other organs.

(2) Various steps of drug transformation may be easily studied using the perfusion technique since the isolated liver preserves its integral cell structure. The relatively simple kinetics of metabolite formation help in the interpretation of the results, overcoming the difficulties met *in vivo* (e.g. the complicated distribution and binding of the drug in the whole body), and *in vitro* (e.g. the damaged functional structures in tissue slices and homogenates).

(3) The technique of liver perfusion offers many possibilities for modifying experimental models, e.g. separate treatment of the donors of liver and blood, modification of the composition of perfusion medium, infusion, dialysis, blood flow changes, etc. Since an isolated liver in good functional condition should be able to metabolize drugs effectively, we were interested in investigating its ability to metabolize drugs after different periods of perfusion. The activity and stability of mixed oxygenases responsible for *O*-demethylation, *N*-demethylation and hydroxylation were examined in the 9000 *g* and microsomal fractions prepared from the perfused livers.

MATERIALS AND METHODS

Liver perfusion. Male Sprague–Dawley rats (CD-COBS from Charles River, Italy) weighing 224 ± 10 g were used as donors of liver and blood. The animals were maintained on a laboratory diet (Charles River 4 RF 26) and water *ad lib.*

The rats were anesthetized with a mixture of chloralose (60 mg/kg of body wt) and sodium phenobarbital (50 mg/kg of body wt), administered intravenously. The liver was isolated by the usual surgical technique and the biliary duct was cannulated.[2] About 3–5 min elapsed between cannulation of the portal vein and completion of surgery, when perfusion was started in the apparatus. The average weight of rat livers was 9·86 ± 0·15 g.

The perfusion medium contained 1/3 homologous defibrinated heparinized blood, 1/3 rat serum and 1/3 Krebs–Ringer bicarbonate buffer pH 7·4 with 0·1% glucose. The perfusate was saturated with a counter current stream of a moistened mixture of 95% O_2 and 5% CO_2 in a thin-film oxygenator. The total volume of the medium was proportional to liver weight, i.e. 4 ml/g liver tissue. The isolated organ was perfused at 37·5° by recirculation for time periods from 30 to 240 min. The flow of the medium was 1 ml/min per g of the organ at 40–50 pulsations/min of the roller pump. Portal pressure was between 80 and 150 mm of water. Bile was collected and its volume was measured.

For the infusion experiments nicotinic acid was dissolved in physiological salt solution (1 mg/ml), neutralized and infused directly into the portal vein cannula at a rate 1·2 ml/hr.

The perfusion apparatus, consisting of a soft liver container, a sampler, a rotating thin-film oxygenator, a roller pump and two thermostated containers of perfusion medium was designed in our laboratory (see Ref. 3 for further details).

Preparation of microsomal fractions. The perfused livers were weighed, placed on dry ice and stored for several days at −20°. The collected organs were homogenized (with a motor driven homogenizer with a Teflon pestle) in 4 vol chilled 1·15% KCl solution, buffered to pH 7·4 with 0·05 M phosphate buffer. The homogenate was centrifuged at 9000 *g* at 5° for 20 min to sediment the nuclei and mitochondria. The supernatant was decanted and used either directly for the determination of the enzymatic activity or for the preparation of microsomes; 2·5 ml of this supernatant corresponded to 500 mg of liver tissue.

Microsomes were prepared from the 9000 *g* fraction by centrifugation at 105,000 *g* for 30 min at 5°. The sedimented microsomal pellet was washed with the buffered KCl and recentrifuged. The washed microsomes were resuspended in the KCl solution; 2·5 ml of this suspension were equivalent to 500 mg of liver tissue and contained 38·2 ± 1·5 mg of protein.

Assays. The incubation mixture contained the substrate, 1·5 μmoles of NADP, 50 μmoles of glucose-6-phosphate, 25 μmoles of $MgCl_2$, 50 μmoles of nicotinamide, 0·08 M potassium phosphate buffer pH 7·0 and the 9000 *g* fraction (2·5 ml) in a total volume of 5 ml; glucose-6-phosphate dehydrogenase (0·5 i.u.) was added when the activity of washed microsomes was measured. Moreover, addition of 1·5 μmoles of NAD, 70 μmoles of ethanol and 0·5 i.u. of alcohol dehydrogenase was necessary for the assay of *N*-demethylation by microsomes.

The following concentrations of substrates were used: aniline 5 μM, *p*-nitroanisol 1·5 μM and aminopyrine 5 μM. The hydroxylation of aniline and the *O*-demethylation of *p*-nitroanisol was determined by the method of Gilbert and Goldberg;[4] the *N*-demethylation of aminopyrine was measured according to the method of LaDu.[5]

The incubation was performed in a shaker at 140 oscillations/min at 37° in air for a period of 30 min.

The cytochrome P_{450} content of the microsomes was measured on a double beam Perkin–Elmer spectrophotometer by the method of Omura and Sato,[6] using a molar extinction coefficient of 91 cm^{-1} mM^{-1} between 450 and 490 nm.

Protein concentrations were determined by the method of Lowry[7] using bovine serum albumin as standard.

For the induction of liver microsomal enzymes phenobarbital (50 mg/kg of body wt) was administered i.p. twice daily for 4 days.

RESULTS

Changes of metabolic activity of the 9000 g *fraction during perfusion of isolated liver*

The activity of liver microsomal enzymes was measured during the perfusion of isolated rat livers. The organs were collected after various intervals of perfusion (30, 60, 120 and 240 min) and homogenized in KCl solution. The 9000 *g* fraction was prepared and the activities were expressed in nmoles/30 min per g of liver tissue. The hydroxylation of aniline and *O*-demethylation of *p*-nitroanisol did not decrease substantially, the activity being about 90 per cent of that of the non-perfused control liver. However, the *N*-demethylation of aminopyrine decreased to 66 per cent with respect to the controls after 30 min of perfusion and to 40 per cent after 4 hr of perfusion (see Table 1).

Effect of nicotinic acid. As may be seen from Table 1, after 2 hr perfusion the *N*-demethylation of aminopyrine by the 9000 *g* fraction of liver decreased to 51 per cent of the activity found in the non-perfused controls. We have examined how addition of nicotinic acid to the perfusion medium affects the 9000 *g* fraction, as this compound is converted very rapidly to NAD by the liver. The infusion of nicotinic acid (1·2 mg/hr) during 2 hr liver perfusion partially restored the *N*-demethylation activity of the 9000 *g* fraction, so that it reached 74 per cent of that found in the non-perfused control organs (see Table 2).

Cytochrome P_{450}. The marked differences between the metabolic activities of the 9000 *g* fraction provoked by liver perfusion led us to check the concentration of microsomal cytochrome P_{450}. The liver microsomes were prepared both from non-perfused controls and from livers perfused for 4 hr. The concentration of cytochrome P_{450} remained the same in both preparations, being 0·650 ± 0·017 nmoles/mg of microsomal protein.

TABLE 1. METABOLIC ACTIVITY OF 9000 *g* FRACTION PREPARED FROM PERFUSED LIVER

Interval of liver perfusion (min)	Substrate metabolized by 9000 *g* fraction [n-moles/30 min per *g* of liver tissue (mean ± S.E.)]*					
	Aniline	(%)	*p*-Nitroanisol	(%)	Aminopyrine	(%)
Non-perfused	496·4 ± 37·1	100·0	511·9 ± 80·9	100·0	422·8 ± 16·5	100·0
30	474·9 ± 22·0	95·5	495·4 ± 35·6	97·0	279·3 ± 22·6†	66·0
60	433·7 ± 25·2	87·0	441·0 ± 21·4	86·0	241·4 ± 25·1†	57·0
120	410·9 ± 30·4	82·5	472·7 ± 20·5	92·5	218·5 ± 10·9†	51·5
240	441·4 ± 48·6	89·0	501·9 ± 40·7	98·0	165·7 ± 10·5†	39·0

* Mean of five animals.

† $P < 0·01$ compared to "non-perfused".

TABLE 2. EFFECT OF NICOTINIC ACID ON METABOLIC ACTIVITY OF PERFUSED LIVER

Rat liver	Aminopyrine metabolized by 9000 *g* fraction (nmoles/30 min per g of liver tissue) (mean ± S.E.)*	(%)
Non-perfused	422·8 ± 16·5	100·0
Perfused 120 min	218·5 ± 10·9	51·5
Perfused 120 min with infusion of nicotinic acid†	312·4 ± 22·4‡	74·0

* Mean of five animals.
† Infusion of nicotinic acid 1·2 mg/hr.
‡ $P < 0.01$ compared to "perfused 120 min".

Effect of phenobarbital induction

The different activities of the 9000 *g* fraction were measured in non-induced, non-perfused control livers and compared with the values found after phenobarbital induction (4 days, 50 mg/kg, twice daily). The phenobarbital treated livers were divided into three groups: (a) induced, non-perfused; (b) induced, perfused for 2 hr; (c) induced, perfused with an infusion of nicotinic acid.

Table 3 shows that all the activities of the 9000 *g* fraction increased after phenobarbital induction, as would be expected. Liver perfusion or infusion with nicotinic acid did not change the hydroxylation of aniline and *O*-demethylation of *p*-nitroanisol. On the other hand, perfusion depressed (group C) the *N*-demethylation of aminopyrine almost to the level found in the non-induced control organs. Infusion of nicotinic acid during the 2-hr perfusion restored this metabolic capacity almost to the level found in induced non-perfused livers.

Effects of NAD and NADP on microsomal activities in perfused and non-perfused livers

We were interested in observing the activity of washed microsomes, their cofactor requirements and the effects of previous liver perfusion. The results of this study are summarized in Table 4. The hydroxylation of aniline, *O*-demethylation of *p*-nitroanisol and *N*-demethylation of aminopyrine were measured either in the presence of

TABLE 3. EFFECT OF PHENOBARBITAL INDUCTION OF METABOLIC ACTIVITY OF PERFUSED LIVER

Rat liver	Substrate metabolized by 9000 *g* fraction (nmoles/30 min per g of liver tissue) (mean ± S.E.)* Aniline	(%)	*p*-Nitroanisol	(%)	Aminopyrine	(%)
1 Non-induced Non-perfused	429·3 ± 20·4	100	451·7 ± 21·6	100	414·7 ± 40·7	100
2 Induced Non-perfused	917·1 ± 43·5†	214	735·4 ± 7·5†	163	638·4 ± 30·1†	154
3 Induced perfused 120 min	911·1 ± 72·6†	212	719·8 ± 38·3†	160	489·5 ± 58·3†	118
4 Induced perfused 120 min + nicotinic acid‡	935·3 ± 63·9†	218	742·8 ± 26·7†	164	599·8 ± 74·3†	144

* Means of five animals.
† $P < 0.01$ for aniline and *p*-nitroanisol 2, 3, 4 vs. 1; for aminopyrine 3 vs. 2.
‡ Infusion of nicotinic acid 1·2 mg/hr.

TABLE 4. EFFECT OF NAD-NADP (0·3 mM ADDED TO MICROSOMES) ON MICROSOMAL ACTIVITIES OF PERFUSED AND NON-PERFUSED RAT LIVER

	Coenzymes added	Substrate metabolized by microsomes (nmoles/30 min per mg of protein) (mean ± S.E.)* Aniline	*p*-Nitroanisol	Aminopyrine
Non-perfused liver	NAD + NADP	2·83 ± 0·01	1·96 ± 0·24	3·85 ± 0·33
	NADP	4·06 ± 0·40	3·40 ± 0·10	3·24 ± 0·17
Activity ratio†		0·69	0·57	1·19
Perfused liver 120 min	NAD + NADP	3·41 ± 0·34	2·12 ± 0·10	3·93 ± 0·50
	NADP	4·92 ± 0·40	3·62 ± 0·20	3·12 ± 0·31
Activity ratio†		0·69	0·58	1·26

* Mean of four values.
† Activity ratio = (NAD + NADP/NADP).

NADPH or of NADH + NADPH (and their respective transferring dehydrogenases).

The activities catalyzed by both NADP + NADPH and/or by NADH were compared and their ratio [NADH + NADPH/NADPH] was calculated.

It should be underlined that this activity ratio did not change substantially (see Table 4) when the microsomes were prepared either from non-perfused control livers or from organs perfused for 2 hr. This is particularly evident when the *N*-demethylation of aminopyrine by washed microsomes is compared with similar data for the 9000 *g* fraction (see Table 1).

The hydroxylation of aniline and the *O*-demethylation of *p*-nitroanisol are inhibited by the presence of NADH in the incubation mixture as shown by an activity ratio lower than 1. On the other hand the *N*-demethylation increases in the presence of NAD, the activity ratio being higher than 1 (see Table 4).

TABLE 5. EFFECT OF NICOTINIC ACID ADDED TO LIVER MICROSOMES, ON LIVER MICROSOMAL ENZYME ACTIVITY

	Substrate metabolized by microsomes (nmoles/30 min per mg of protein) Aniline	*p*-Nitroanisol	Aminopyrine
NAD + NADP* Nicotinic acid†	3·77	2·74	4·44
NAD + NADP* Control	4·05	3·02	5·09
Activity ratio‡	0·93	0·91	0·87
NADP* Nicotinic acid†	5·52	4·35	3·61
NADP* Control	5·27	4·22	3·61
Activity ratio‡	1·05	1·03	1·00

* NAD and NADP concentration 0·3 mM.
† Nicotinic acid concentration 0·2 mM.
‡ Activity ratio = (Nicotinic acid/Control).

Direct effect of nicotinic acid on microsomal activity

Microsomes were incubated with the usual substrates and cofactors (NAD + NADP or NADP) in the presence of nicotinic acid (0·2 mM). It can be demonstrated that nicotinic acid has either a slightly inhibitory or no effect on the metabolic activity of washed microsomes (see Table 5).

DISCUSSION

The perfusion technique is often used to study drug metabolism, but little attention has been paid to the changes in metabolic activity of the isolated organ. The prolonged perfusion may be considered as a continuous depletion of the internal resources of liver tissue (e.g. respiratory coenzymes) which influences also the rates of drug transformation. However, the observed decrease of microsomal activity is not uniform, but depends on the type of substrate. This suggests the importance of further analysis of these changes considering also other possible applications of liver perfusion technique (e.g. organ conservation).

The metabolism of three different model substrates was studied in the 9000 *g* and/or microsomal fractions prepared from livers perfused for various intervals. It was noted that the rates of aniline hydroxylation and of *O*-demethylation of *p*-nitroanisol did not diminish substantially after 4 hr of liver perfusion.

Whereas the rate of *N*-demethylation of aminopyrine decreased markedly, being reduced to 40 per cent of the initial activity found in non-perfused liver.

Similar results were obtained after the induction of liver donors by phenobarbital. This treatment increased the overall activity of the 9000 *g* fraction prepared from non-perfused livers, but the perfusion of the isolated organ for 2 hr decreased the rate of *N*-demethylation almost to the values found in non-induced non-perfused livers.

Moreover, it is evident that the conditions under which liver perfusion is carried out are no less important than the type of reaction studied. Bock *et al.*[8] observed that hexobarbital metabolism was reduced both when livers were isolated from starved rats and when an erythrocyte-free medium was used for organ perfusion. This decrease of metabolic rate may be considered as a consequence of the limited supply of nicotinamide nucleotides and oxygen under these conditions.

The NAD (P) glycohydrolase (E.C. 3.2.2.6.) is considered responsible for the degradation of both NAD and NADP. It has been suggested that *in vivo* NAD (P) glycohydrolase is inactive.[10] On the other hand in the liver perfused with erythrocyte free medium the activity of NAD glycohydrolase increased by 35 per cent.[8] This activation of NAD glycohydrolase has been observed also in tuberculous livers[11] and after starvation.[12]

The availability of nicotinamide nucleotides controls the directions of cell metabolism and forms an important link between the different competing metabolic pathways. The steady-state concentration of these nucleotides, as of other cell constituents, is maintained as a consequence of enzymatic synthesis and breakdown.

Biosynthesis of NAD and NADP may occur by three routes: from nicotinic acid, from nicotinamide and from tryptophan.[10] Nicotinic acid infused in low concentration during liver perfusion is a more effective precursor of NAD than nicotinamide; large doses of nicotinic acid inhibit NAD biosynthesis in the perfused liver.[13]

In our experiments the infusion of low doses of nicotinic acid (10 μmoles/hr) during liver perfusion partly restores the decreased *N*-demethylation of aminopyrine,

both in normal and in phenobarbital induced and perfused livers. The increased availability of NAD produced from nicotinic acid very probably counterbalances the depletion of NAD due to the enhanced glycohydrolase activity during perfusion of the isolated liver. The addition of nicotinic acid to the incubation medium has no marked effect on microsomal enzyme activity, while the addition of NAD increases the *N*-demethylation of aminopyrine, but inhibits the transformation of aniline and *p*-nitroanisol.

The importance of cofactor availability is underlined by the finding that the concentration of cytochrome P_{450} remained constant even after 4 hr of liver perfusion. The value obtained in our experiments was lower than that found by Bock *et al.*[8]

In conclusion, the importance of the accurate control of experimental conditions when the technique of perfusion of isolated liver is used for studies on drug metabolism must be stressed. These results underline the problem that lack of necessary cofactors may change the parameters of drug transformation.

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