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Evidence that Ligand Formation is a Mechanism underlying the Maintenance of Cytochrome *P*-450 in Rat Liver Cell Culture

POTENT MAINTENANCE BY METYRAPONE

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The loss of cytochrome *P*-450 in cultured rat hepatocytes can be prevented by substituted pyridines, especially isonicotinamide, 3-hydroxypyridine and metyrapone. The effect of these compounds is independent of protein synthesis, suggesting that they maintain pre-existing cytochrome *P*-450. The efficiency of pyridines at maintaining cytochrome *P*-450 in hepatocyte culture is highly correlated with their ability to bind to this cytochrome, suggesting that ligand formation with cytochrome *P*-450 prevents its accelerated turnover in liver cell culture.

Rat hepatocytes cultured for 24h lose 70% of their cytochrome *P*-450 content (Guzelian *et al.*, 1977; Paine & Legg, 1978). This spontaneous loss of cytochrome *P*-450 can be prevented by the incorporation of hyperphysiological concentrations of nicotinamide (pyridine-3-carboxylic acid amide) in the culture medium (Paine *et al.*, 1979*a*). Although isonicotinamide (pyridine-4-carboxylic acid amide), a non-physiological isomer of nicotinamide, is twice as efficient as nicotinamide (Paine *et al.*, 1979*b*), and high concentrations of other substituted pyridines, including pyridine itself, will maintain cytochrome *P*-450 in hepatocytes cultured for 24h at the same concentration as found in intact liver (Villa *et al.*, 1980), the mechanism underlying this effect has been unknown until now. The present work shows that the ability of these compounds to form ligands with cytochrome *P*-450 is correlated with their efficiency at maintaining this cytochrome. This suggests that ligand formation is a mechanism underlying the maintenance of cytochrome *P*-450 in rat liver cell culture.

Materials and Methods

Isolation and culture of hepatocytes

Hepatocytes were isolated and cultured as previously described (Paine *et al.*, 1979*b*).

The compounds studied were dissolved directly in the culture medium and were used without filter sterilization.

Cytochrome P-450 and protein

These were determined as previously described (Paine *et al.*, 1979*b*).

Protein synthesis

This was determined by measuring the incorporation of L-[U-^{14}C]leucine into trichloroacetic acid-insoluble material as previously described (Villa *et al.*, 1980).

Preparation of microsomal fraction

Adult male rats of the Porton-derived Wistar strain fed *ad libitum* on MRC 41B diet were used without treatment or were given 1mg of phenobarbitone (BDH, Poole, Dorset, U.K.)/ml of drinking water for at least 7 days before use. The rats were decapitated and the liver microsomal fractions were prepared as described by McLean & Day (1974). The cytochrome *P*-450 content of microsomal preparations from control and phenobarbitone-treated rats was 0.87 and 1.7nmol/mg of protein respectively.

Binding spectra

These were recorded on a Unicam SP.8-100 spectrophotometer with oxidized microsomal suspensions (2.7ml, 1.3–2mg of microsomal protein) in each 1cm-light-path cuvette. Difference spectra ($A_{420}-A_{500}$) were recorded after addition of the

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compound under study dissolved in water (5–200 μl) to the sample cuvette and an equal volume of water to the reference cuvette. The final concentrations in the cuvette of the compounds studied were varied from 0.5 to 20 μM for metyrapone, 60 μM to 3 mM for pyridine, 50 μM to 7 mM for 3-acetylpyridine, 50 μM to 6 mM for 3-aminopyridine, 50 μM to 3 mM for isonicotinamide, 50 μM to 3 mM for 3-hydroxypyridine and 250 μM to 11 mM for nicotinamide. K_s (binding affinity constant) values for each compound were computed from a double-reciprocal plot of $A_{420}-A_{500}$ versus concentration (Schenkman *et al.*, 1967) for at least seven different concentrations by linear regression by using the method of least squares. The K_s computed was 0.95 μM for metyrapone, 0.22 mM for 3-hydroxypyridine, 0.24 mM for 3-acetylpyridine, 0.74 mM for isonicotinamide, 0.22 mM for pyridine and 2.25 mM for nicotinamide.

Statistics

Results are quoted as means ± S.D. and were analysed by Student's *t* test. The correlation coefficient between K_s and the concentration of compound to maintain cytochrome *P*-450 in hepatocyte culture was computed by linear regression by using the method of least squares.

Chemicals

Isonicotinamide, 3-acetylpyridine, 3-aminopyridine, 3-hydroxypyridine, hexahydronicotinamide and nicotinic acid hydrazide were purchased from Aldrich Chemical Co., Gillingham, Dorset, U.K. Nicotinamide and pyridine (AnalaR) were from BDH. Cycloheximide, nicotinic acid and isonicotinic acid hydrazide were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Metyrapone (Metopirone, 'free base pure substance') was a generous gift from Ciba Laboratories, Horsham, Sussex, U.K.

Results and Discussion

Previous work has shown that the sole addition of 25 mM-nicotinamide, 25 mM-pyridine, 10 mM-3-acetylpyridine, 10 mM-3-aminopyridine or 10 mM-isonicotinamide to a cell culture medium maintains high concentrations of cytochrome *P*-450 in hepatocytes cultured for 24 h (Villa *et al.*, 1980). Isonicotinamide was the most efficient of these compounds at maintaining cytochtome *P*-450 and, as shown in Table 1, culture medium containing 10 mM-isonicotinamide maintains cytochrome *P*-450 in hepatocytes cultured for 24 h at a concentration that is not significantly different ($P > 0.05$) from that in freshly isolated cells or intact liver. Although 3-hydroxypyridine is twice as efficient as isonicotinamide at maintaining the cytochrome *P*-450 content of cultured hepatocytes, metyrapone [2-methyl-1,2-di-(3-pyridyl)propan-1-one] is the most potent compound examined so far (Table 1).

The ability of isonicotinamide, 3-hydroxypyridine or metyrapone to maintain cytochrome *P*-450 in liver cell culture is independent of protein synthesis, as culture medium containing 2.5 μM-cycloheximide, which produces a 95% inhibition of protein synthesis in the presence or absence of these compounds, does not prevent the maintenance of cytochrome *P*-450. Culture medium containing 2.5 μM-cycloheximide alone did not result in the maintenance of cytochrome *P*-450. This finding is at variance with that of Guzelian & Barwick (1979), but is in agreement with our previous conclusion that the inhibition of protein synthesis is not a mechanism underlying the maintenance of cytochrome *P*-450 in hepatocyte culture (Villa *et al.*, 1980). This finding suggests that these compounds maintain cytochrome *P*-450 pre-existing in hepatocytes rather than affecting the rate of cytochrome *P*-450 synthesis.

Metyrapone is known to form a high-affinity ligand with cytochrome *P*-450 (Hildebrandt *et al.*, 1969), and therefore a possible common denominator between the ability of substituted pyridines to

Table 1. *Dose–response relationship for the maintenance of cytochrome P-450 by isonicotinamide, 3-hydroxypyridine or metyrapone in hepatocytes cultured for 24 h*

Hepatocytes were cultured for 24 h in Williams medium E containing isonicotinamide, 3-hydroxypyridine or metyrapone at the concentrations shown. Cytochrome *P*-450 was measured as described in the Materials and Methods section. The results are means ± S.D. for individual values found in preparations derived from three separate rat livers (i.e. $n = 3$). The initial cytochrome *P*-450 concentration of freshly isolated hepatocytes was 164 ± 30 ($n = 15$) pmol/mg of protein.

	Cytochrome *P*-450 (% of initial)						
Concn. (mM) of compound studied ...	0	0.25	0.5	1	5	10	15
Compound studied							
Isonicotinamide	32 ± 9	—	—	35 ± 8	59 ± 4	88 ± 9	Cytotoxic
3-Hydroxypyridine	30 ± 4	—	36 ± 3	58 ± 10	106 ± 11	Cytotoxic	—
Metyrapone	32 ± 6	85 ± 10	105 ± 6	89 ± 12	Cytotoxic	—	—

maintain cytochrome *P*-450 in liver cell culture could be their ability to bind to cytochrome *P*-450.

Cytochrome *P*-450, like other haemoproteins, exists in low- and high-spin states which correlate with optical spectra. Nitrogen bases such as pyridine bind to cytochrome *P*-450 producing a low-spin state, which results in a Soret band at 420nm (Jefcoate *et al.*, 1969; Temple, 1971). The magnitude of the absorbance at 420nm is dependent on the ligand concentration, and a double-reciprocal plot of absorbance versus ligand concentration permits the derivation of a binding affinity constant (K_s), which is analogous to K_m derived from a Lineweaver–Burk plot (Schenkman *et al.*, 1967). In Fig. 1 the K_s for the binding to cytochrome *P*-450 in microsomal suspensions found for each substituted pyridine known to maintain cytochrome *P*-450 in hepatocyte culture is plotted as a function of the concentration that produces a 75% maintenance of cytochrome *P*-450, since, unlike isonicotinamide, 3-hydroxypyridine and metyrapone, not all pyridines produce a 100% maintenance of cytochrome *P*-450 after 24h of culture (Villa *et al.*, 1980). Nevertheless Fig. 1 shows that there is a good correlation ($r = +0.94$) between the ability of pyridines to bind to cytochrome *P*-450 in microsomal suspensions and to maintain this cytochrome in liver cell culture. The K_s value for the binding of these compounds to cytochrome *P*-450 in microsomal suspensions was also highly correlated ($r = +0.97$) when plotted as a function of the concentration that produces a 50% maintenance of cytochrome *P*-450 in liver cell culture. These correlations were independent of whether microsomal fractions from control rats ($r = +0.89$) or phenobarbitone-induced rats ($r = +0.94$) were used to compute K_s. Pyridine is the only compound for which there is a poor correlation (Fig. 1). This is probably due to the volatility of pyridine, which invalidates the assumption that its final concentration in the culture medium is the same as the initial concentration. Of all the compounds examined, only hexahydronicotinamide does not form a ligand with cytochrome *P*-450, nor does it maintain this cytochrome in liver cell culture. Substituted pyridines which do not maintain cytochrome *P*-450 in liver cell culture, such as nicotinic acid hydrazide, isonicotinic acid hydrazide and nicotinic acid, do form ligands with cytochrome *P*-450. However, their K_s values (3.2, 1 and 4.9mM respectively) are such that one would predict from Fig. 1 that the concentration required to maintain the cytochrome in liver cell culture would exceed their respective cytotoxic concentrations (Villa *et al.*, 1980).

We therefore conclude from these findings and from the observation that protein synthesis is apparently not required for the maintenance of cytochrome *P*-450 that ligand formation may stabilize cytochrome *P*-450 in cultured hepatocytes and prevent its accelerated turnover.

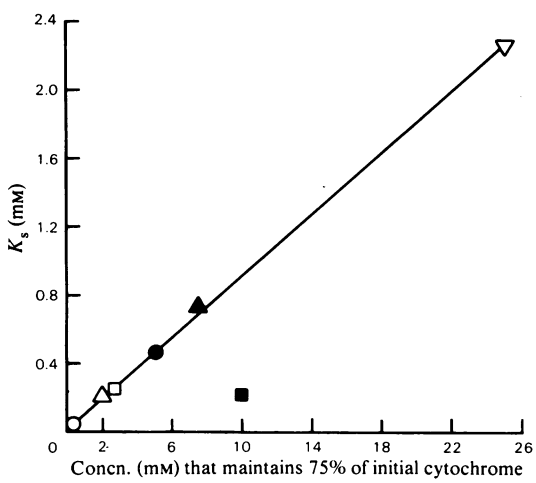

Fig. 1. *Correlation between binding affinity constant (K_s) of pyridines to cytochrome P-450 and their efficiency to maintain this cytochrome in hepatocytes cultured for 24 h*

K_s values were computed as described, and are given, in the Materials and Methods section. The concentrations required to maintain 75% of the initial cytochrome *P*-450 in hepatocytes cultured for 24h are taken from the dose–response relationships shown in Table 1 and from Villa *et al.* (1980). ○, Metyrapone; △, 3-hydroxypyridine; □, 3-acetylpyridine; ●, 3-aminopyridine; ▲, isonicotinamide; ■, pyridine; ▽, nicotinamide.

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