Relationship between the Ability of Nicotinamide to Maintain Nicotinamide–Adenine Dinucleotide in Rat Liver Cell Culture and its Effect on Cytochrome *P*-450

By Alan J. PAINE, Lesley J. HOCKIN and Robert F. LEGG
M.R.C. Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

(Received 15 August 1979)

Rat hepatocytes cultured for 24h lose 60% of their NAD content. Treatment with nicotinamide prevents the loss of NAD as well as the previously reported loss of cytochrome *P*-450, suggesting a possible causal relationship. However, isonicotinamide also prevents the loss of cytochrome *P*-450, but does not increase the concentration of NAD, demonstrating that the ability of nicotinamide to maintain cytochrome *P*-450 is not apparently related to its effect on the NAD content of cultured hepatocytes.

The primary culture of hepatic parenchymal cells results in the spontaneous loss of 70% of their cytochrome *P*-450 during the first 24h (Guzelian *et al.*, 1977; Paine & Legg, 1978). This spontaneous loss can be prevented by the inclusion of high unphysiological concentrations of nicotinamide in the culture medium (Paine *et al.*, 1979a). However, the mechanism of this effect is unknown.

An obvious consequence of nicotinamide administration to experimental animals is to increase the hepatic concentration of NAD (Blake *et al.*, 1967). Accordingly we have examined the relationship between the abilities of nicotinamide and isonicotinamide to maintain cytochrome *P*-450 and their effects on the content of nicotinamide coenzymes in cultured hepatocytes. Three conclusions result from this study.

(1) The main conclusion is that rat liver cells cultured for 24h lose 60% of their NAD content. This previously unreported finding must be taken into account when hepatocyte cultures are used to examine biochemical mechanisms at the cellular level.

(2) The loss of NAD may be overcome by supplementing culture medium with nicotinamide, which maintains NAD in cultured hepatocytes at the same concentration as found in intact rat liver.

(3) Treatment with nicotinamide also prevents the loss of cytochrome *P*-450 from cultured hepatocytes, but this is shown to be unrelated to its effect on NAD.

Materials and Methods

Isolation of hepatocytes

Adult (180–250g) male rats of the Porton-derived Wistar strain, fed *ad libitum* on MRC 41B diet, were anaesthetized by intraperitoneal injection with

60mg of pentobarbitone/kg. Hepatocytes were then isolated by perfusion of the liver with 0.05% (w/v) collagenase (Worthington type II, supplied by Cambrian Chemicals, Croydon, Surrey, U.K.) in Ca^{2+} - and Mg^{2+} -free Hanks balanced salt solution (Gibco Biocult, Paisley, Scotland, U.K.) as previously described (Paine *et al.*, 1979b). Perfusions were commenced between 10:30 and 11:30h. This process yielded approx. 5×10^8 parenchymal cells per liver, depending on the size of rat used, with a viability of $88 \pm 6\%$ (mean $\pm$ s.d. for 18 perfusions), as assessed by Trypan Blue exclusion.

Culture of cells

Hepatocytes were cultured at a density of 18×10^6 cells/20ml of medium in 150mm-diameter Petri dishes (Lux Scientific, supplied by Gateway International, Middlesbrough, Cleveland, U.K.). The medium used was Williams medium E with or without nicotinamide or isonicotinamide and supplemented with 5% (v/v) foetal calf serum and 5mg of gentamicin/100ml (medium and supplements were purchased from Flow Laboratories, Irvine, Scotland, U.K.). Cells were cultured at 37°C under a humidified atmosphere of 5% CO_2 in air.

Biochemical determinations

These were carried out on cultured hepatocytes after the culture medium had been aspirated and the cell monolayer had been washed with ice-cold 0.15M-NaCl (2×30 ml).

Cytochrome *P*-450 content of intact liver, isolated cells and hepatocytes cultured for 24h, was determined by recording on a Unicam SP.8–100 spectrophotometer (1 cm light path) the difference spectrum of reduced homogenate plus CO versus reduced

homogenate, by taking the precautions previously described (Paine & Legg, 1978). For this determination the samples used were homogenized in 20% (v/v) glycerol in 0.1 M-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA and 0.2% (w/v) emulgen (Sinclair *et al.*, 1979) by ten up-and-down strokes of a glass homogenizer fitted with a Teflon pestle, with a clearance of 0.5 mm and driven at 2000 rev./min. The final concentration of tissue used to record the difference spectrum was 20 mg of liver/ml or 3–5 mg of isolated or cultured cell protein/ml.

Nicotinamide coenzymes were determined spectrophotometrically as described by Klingenberg (1974). For the determination of the nicotinamide coenzyme concentration in intact rat liver, rats were anaesthetized with pentobarbitone exactly as described under 'Isolation of hepatocytes', except that upon opening the abdomen the left lateral lobe of the liver was freeze-clamped with tongs cooled to the temperature of liquid N₂. The frozen portion of liver was then ground in liquid N₂ with a mortar and pestle. Then 1 g samples of powdered liver or 200 × 10⁶ isolated hepatocytes were added to 5 ml of 0.6 M-HClO₄ for the acid extraction of NAD⁺ and NADP⁺ and to 5 ml of 0.5 M-KOH in 50% (v/v) ethanol in water precooled to -15°C for the alkaline extraction of NADH and NADPH. Hepatocytes (approx. 10⁸ cells) cultured for 24 h were scraped from 6 × 150 mm-diameter Petri dishes into either 5 ml of 0.5 M-KOH in aq. 50% (v/v) ethanol or into 5 ml of 50 mM-Tris/HCl/0.15 M-KCl to which 3 M-HClO₄ was added to give a final concentration of 0.6 M-HClO₄. NAD⁺ in these extracts was determined by the action of alcohol dehydrogenase (EC 1.1.1.1) on ethanol, NADP⁺ by glucose 6-phosphate dehydrogenase (EC 1.1.1.49) on glucose 6-phosphate, NADH by lactate dehydrogenase (EC 1.1.1.27) on pyruvate and NADPH by

glutamate dehydrogenase (EC 1.4.1.3) on oxoglutarate. The addition of 200 nmol of NAD⁺, NADP⁺, NADH and NADPH to such extracts showed that the recovery of individual nucleotides was 95%. Individual nucleotides, as well as all substrates and enzymes for their determination, were of the highest purity available from Boehringer Corp., Bell Lane, Lewes, Sussex, U.K.

Protein was assayed by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (Sigma Chemical Co., Poole, Dorset, U.K.) as the standard.

Other chemicals

Nicotinamide and isonicotinamide (4-pyridine-carboxylic acid amide) were purchased from BDH, Poole, Dorset, U.K. and Aldrich Chemical Co., Gillingham, Dorset, U.K. respectively. Emulgen 913 was from the Kao Atlas Co., Tokyo, Japan. All other chemicals were the highest purity from BDH.

Results and Discussion

Isolated hepatocytes are known to lose low-molecular-weight constituents during their preparation (see Viña *et al.*, 1978). However, Table 1 shows that the total nicotinamide-adenine nucleotide concentration of freshly isolated cells is not significantly different from that found in freeze-clamped liver, and, although these are lower than those reported by Bergmeyer (1974), they are in agreement with values previously found in the livers of Porton rats (Threlfall, 1959). In contrast with isolated hepatocytes, cells cultured for 24 h without treatment lose 60% of their total nucleotide content, which is due to a loss of NAD⁺ plus NADH (referred to as NAD throughout) (Table 1). Although the loss of nicotinamide coenzymes has been proposed

Table 1. Effect of nicotinamide and isonicotinamide on the concentration of nicotinamide coenzymes and cytochrome P-450 in hepatocytes cultured for 24 h

Samples of freeze-clamped liver, isolated hepatocytes and rat liver cells cultured for 24 h in Williams medium E plus or minus nicotinamide or isonicotinamide at the concentrations shown were prepared and their cytochrome P-450 and nicotinamide coenzyme concentrations assayed 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 (namely *n* = 3). The initial cytochrome P-450 concentration of freshly isolated hepatocytes was not significantly different from that found in freeze-clamped liver and was 188 ± 27 (*n* = 23) pmol/mg of protein.

Treatment and sample origin	Cytochrome P-450 (% of initial)	Nucleotide concn. (nmol/mg of protein)		
		NAD ⁺ + NADH	NADP ⁺ + NADPH	Total
Freeze-clamped liver	100	2.6 ± 0.4	0.8 ± 0.1	3.4 ± 0.3
Isolated hepatocytes	100 ± 16	3.2 ± 0.2	1.1 ± 0.6	4.3 ± 0.9
Hepatocytes cultured for 24 h				
(a) No treatment	26 ± 11	0.9 ± 0.3	0.8 ± 0.3	1.7 ± 0.5
(b) +2 mM-Nicotinamide	21 ± 8	1.9 ± 0.2	1.3 ± 0.1	3.2 ± 0.4
(c) +25 mM-Nicotinamide	74 ± 3	2.1 ± 0.1	1.4 ± 0.05	3.5 ± 0.03
(d) +10 mM-Isonicotinamide	100 ± 10	0.7 ± 0.2	0.3 ± 0.1	1.0 ± 0.3

(Schoental, 1975) to occur in liver cell culture, it has not to the best of our knowledge been shown until now. In agreement with findings in experimental animals (Blake *et al.*, 1967), treatment with nicotinamide markedly increases the NAD content of liver cells, such that after 24h of culture in medium containing either 2mM or the high unphysiological concentration (25mM) of nicotinamide the NAD and total nicotinamide-adenine nucleotide content of cultured hepatocytes is not significantly ($P>0.05$) different from that in freeze-clamped liver. The finding that culture medium containing 2mM-nicotinamide will maintain NAD at the same concentration as medium containing 25mM-nicotinamide, whereas only the latter will prevent the loss of cytochrome *P*-450 (Table 1), suggests that there is not a causal relationship between the content of NAD and the maintenance of cytochrome *P*-450 in cultured rat hepatocytes. This is confirmed by the finding that isonicotinamide is twice as efficient as nicotinamide at maintaining the content of cytochrome *P*-450, yet isonicotinamide does not increase the content of NAD (Table 1). Although the mechanism of action of nicotinamide remains to be determined, the present work clearly shows that the ability of nicotinamide to maintain cytochrome *P*-450 is not related to its ability to increase the nucleotide content of cultured hepatocytes.

References

- Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 2298–2299, Academic Press, London and New York
- Blake, R. L., Blake, S. L., Loh, H. H. & Kun, E. (1967) *Mol. Pharmacol.* **3**, 412–422
- Guzelian, P. S., Bissell, D. M. & Meyer, U. A. (1977) *Gastroenterology* **72**, 1232–1239
- Klingenberg, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 4, pp. 2045–2058, Academic Press, London and New York
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Paine, A. J. & Legg, R. F. (1978) *Biochem. Biophys. Res. Commun.* **81**, 672–679
- Paine, A. J., Williams, L. J. & Legg, R. F. (1979a) *Life Sci.* **24**, 2185–2192
- Paine, A. J., Williams, L. J. & Legg, R. F. (1979b) in *The Liver: Quantitative Aspects of Structure and Function* (Preisig, R., Bircher, J. & Paumgartner, G., eds.), vol. 3, Editio Cantor, Aulendorf, in the press
- Schoental, R. (1975) *Biochem. Soc. Trans.* **3**, 1033–1034
- Sinclair, J. F., Sinclair, P. R. & Bonkowsky, H. L. (1979) *Biochem. Biophys. Res. Commun.* **86**, 710–717
- Threlfall, C. J. (1959) *Nature (London)* **184**, 60–61
- Viña, J., Hems, R. & Krebs, H. A. (1978) *Biochem. J.* **170**, 627–630