Biochemical Pharmacology, Vol. 25, pp. 281–283. Pergamon Press, 1976. Printed in Great Britain.

THE METABOLISM OF [^{14}C]NICOTINE BY ISOLATED RHESUS MONKEY HEPATOCYTES *IN VITRO*

ALAN POOLE and COLIN URWIN

Department of Cell Biology, Huntingdon Research Centre, Huntingdon, Cambs., England, PE18 6ES

(*Received* 24 *May* 1975; *accepted* 24 *July* 1975)

Abstract—The *in vitro* metabolism of [^{14}C]nicotine was investigated using isolated rhesus monkey (*Macaca mulatta*) hepatocytes. Under aerobic conditions the cells metabolised nicotine to the *N*-oxide, cotinine and other compounds which were not identified. Nicotine and its metabolites were demonstrated in the growth medium and within the cells showing the latter to maintain an excretory function.

Mammalian cell cultures can provide test systems for the biochemical investigation of metabolism within the intact cell. In recent years a variety of methods have been used to prepare isolated hepatocyte cultures [1–5, 7]. Some authors have known that their methods were of limited application since electron microscopical examination showed, in some cases, marked alterations in the morphological appearance of the cells.

The contamination of hepatocyte cultures with fibroblasts to give mixed cell cultures has also posed problems in the study of hepatocyte function *in vitro*.

The present paper describes a method, for the study of hepatocytes *in vitro*, which inhibits the growth of fibroblasts and preserves the structure and metabolic activity to cotinine and to nicotine-*N*-oxide.

MATERIALS AND METHODS

Livers from adult rhesus monkeys (*Macaca mulatta*) were used. Collagenase and hyaluronidase were obtained from Sigma (London) Chemical Co. Ltd. Medium 199 with Earle's salts, and bovine serum and antibiotics were obtained from Flow Laboratories, Scotland; babies' feeding bottles made from polycarbonate were obtained from Maws Ltd., England. [^{14}C]Nicotine was obtained from Radiochemical Centre, Amersham (sp. act. 10–25 mCi/mM).

Preparation of isolated liver cells. The monkey was anaesthetised with althesin and exsanguinated prior to the removal of the liver. All procedures were performed aseptically. The abdominal cavity was opened by midline incision with lateral extensions and the intestine was moved to the left side. The portal vein was exposed and cannulated. Another cannula was tied in the thoracic portion of the inferior vena cava. To prevent leakage of enzyme from the liver, arterial forceps were used to clamp the hepatic artery and the inferior vena cava anterior to the renal vein. The gall bladder was dissected, and the liver was removed from the body cavity and placed in a sterile container. The liver was perfused according to the method of Berry and Friend [2] with the modification that the perfusion was allowed to continue for three hr. when the liver began to disintegrate. At the termination of perfusion, the liver was placed in a sterile conical flask and shaken with enzyme solution. The resulting cell suspension was filtered through sterile gauze into a centrifuge tube and washed three times in a refrigerated centrifuge at 800 rev/min for 10 min. The washing medium comprised 250 ml of Hanks BSS 5% calf serum, 1% penicillin/streptomycin (100 IU/ml).

The cells were suspended in Medium 199 with Earle's salts supplemented with 10% calf serum and 1% penicillin and streptomycin. The polycarbonate bottles were seeded with 30 ml of suspension, each ml containing 10^5 cells. The bottles were placed on a tissue culture roller apparatus at 12 rev/hr in an incubator at 37°. After 48 hr the old medium was removed and fresh medium added.

Cell culture. When the cells were checked under the microscope 48 hr after inoculation, nearly 60 per cent were found to be attached and flattened. The unattached cells were removed during the medium change and discarded. After 4 days, small numbers of fibroblast-like cells were seen in the polycarbonate bottles. However, by the sixth day these had disappeared. This result is in contrast to that with cells grown on Falcon Petri dishes, in which the fibroblasts appeared after the fourth day, and by the sixth had completely overgrown the culture.

The cells in the polycarbonate bottles appeared not to divide, and by the 30th day no mitotic activity could be detected.

Electron microscopy. After 10 days incubation the cells were fixed in 4% glutaraldehyde in 0·134 M phosphate buffer pH 7·25. They were centrifuged at 1000 rev/min for 10 min and the resulting pellet was post fixed in 1% osmium tetroxide in 0·2 M phosphate buffer pH 7·4, dehydrated in alcohol and embedded in epoxy resin. 1 μm survey sections were cut and stained with 1% toluidine blue for examination with the light microscope. Silver–gold ultra thin sections of representative areas were taken, mounted on copper grids and subsequently stained with lead citrate. The sections were examined using a Phillips EM 300 operating at 60 KV. Selected areas were photographed on Kodak Fine Grain Positive 35 mm film (Fig. 2).

Metabolism of nicotine. [^{14}C]nicotine was added to three cultures after 10 days. The cells were incubated with the nicotine for 30 min, 2 hr and 24 hr. After incubation the cells were harvested using 1%

Table 1. The R_f values of reference compounds and compounds isolated from cells. T.l.c. in ethanol–acetone–benzene–ammonium hydroxide (5:40:50:5)

Reference compound	R_f	Isolated compound	R_f
		Unknown	0·84
Nicotine	0·71	Nicotine	0·71
Cotinine	0·56	Cotinine	0·56
		Unknown	0·18
		Unknown	0·12
Nicotine-*N*-oxide	0·09	Nicotine-*N*-oxide	0·09

trypsin and a centrifuge at 1000 rev/min for 10 min. The pellets of cells were homogenised with chloroform–methanol (2:1). The radioactive components in the chloroform extract were separated by thin-layer chromatography on pre-layered Kieselgel F 254 plates (E. Merck AG) of 0·25 mm thickness using ethanol–acetone–benzene–ammonium hydroxide (5:40:50:5) as the developing solvent. Non-radioactive reference compounds were detected by their quenching of fluorescence at 254 μm.

Radioactive components on thin-layer plates were detected by apposition autoradiography on X-ray film. Zones of radioactivity adsorbed on silica gel and detected by autoradiography were excised and ground to a fine powder. This powder was thoroughly mixed with water and Triton X-100 based scintillator. The technique gives recoveries of radioactivity exceeding 95 per cent.

Table 2. Percentage of radioactive nicotine, cotinine and nicotine-*N*-oxide in cells at 30 min, 2 hr and 24 hr.

Time	Nicotine	Cotinine	Nicotine-*N*-oxide	Unknown
30 min	98·9	0·7	0·3	0
2 hr	96·4	2·7	0·7	0·1
24 hr	94	4·6	1·15	0·25

RESULTS

Morphology. The ultrastructure of the hepatocytes maintained *in vitro* for 10 days and observed by electron microscopy was very similar to that of those which had been freshly removed. No morphological differences were apparent in the cell membrane, Golgi complex, rough- and smooth-surfaced endoplasmic

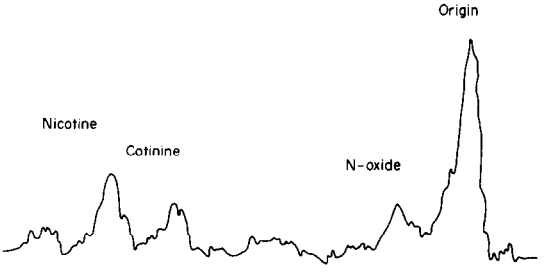

Fig. 1. Scan of thin-layer chromatogram of chloroform phase of extract of hepatocytes obtained from the rhesus monkey (*Macaca mulatta*) cells incubated with [^{14}C]nicotine 5 μCi/cm^3 in growth medium for 24 hr prior to extraction. Solvent system ethanol–acetone–benzene–conc. NH_4OH (5:40:50:5).

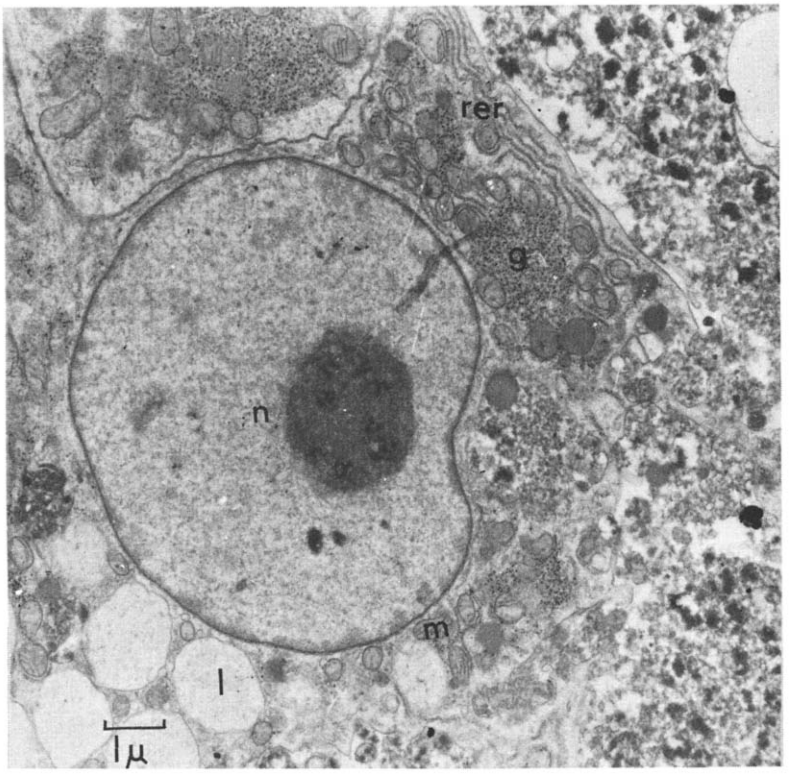

Fig. 2. Electron micrograph of hepatocyte after 10 days in culture. n—nucleus, g—glycogen, m—mitochondria, l—lipid, rer—rough endoplasmic reticulum show no apparent changes in morphology. Mag × 10,800.

reticulum, mitochondria, microbodies, lysosomes and nuclei when compared with those of normal hepatocytes. Some groups of cells aggregated to form bile canalicular-like spaces.

Detection and identification of metabolites in cells. The R_f values (relative fractions) of reference compounds used in conjunction with thin-layer chromatography (t.l.c.) of the cells extracts are shown in Table 1 (The R_f of metabolites characterised by t.l.c. from cells are also shown). The amounts of the individual components nicotine, cotinine and nicotine *N*-oxide in the cell extracts at 30 min, 2 hr and 24 hr are shown in Table 2.

The major metabolite detected by t.l.c. after each time period was identical to cotinine. There were smaller amounts of nicotine-*N*-oxide. Several minor metabolites were observed, but reference compounds were not available for their identification (Fig. 1).

DISCUSSION

The major advantage of the technique described in this paper is the successful maintenance *in vitro* of adult mammalian hepatocytes without the usual overgrowth of the culture by fibroblasts.

Examination by electron microscopy of the hepatocytes after 10 days in culture showed that they retained their structural integrity.

No mitotic figures were observed in hepatocytes in cultures when examined microscopically at daily intervals.

It was found that hepatocytes cultured by the described technique were capable of metabolising [^{14}C]nicotine and releasing some metabolites into the growth medium. Of the nicotine metabolites, cotinine was by far the most important in a quantitative sense, although nicotine-*N*-oxide and the other unknown compounds appeared regularly in all the experiments performed with hepatocytes *in vitro*. Attempts to identify the unknown compounds ($R_f = 0{\cdot}12$) ($R_f = 0{\cdot}18$) and ($R_f = 0{\cdot}84$) were unsuccessful; however it was suspected that ($R_f = 0{\cdot}12$) is hydroxycotinine and that ($R_f = 0{\cdot}18$) is γ-(3-pyridyl)-γ-oxo-*N*-methyl butyramide. Further work will be needed to determine the nature of the unidentified fractions.

This study demonstrates that primary hepatocyte cultures may be maintained in culture for relatively long periods (up to 35 days; unpublished work). The cells retain their ultrastructural resemblance to intact hepatocytes and their ability to metabolise micotine to cotinine and nicotine-*N*-oxide which are major metabolites *in vivo* [6].

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