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FATE OF THE MONOAMINE OXIDASE INHIBITOR PARGYLINE IN CULTURED HEPATOCYTES

ANGELA M. MAYNE and PETER J. EVANS*
Department of Applied Biology, UWIST, King Edward VII Avenue, Cardiff CF1 3XF, U.K.

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Abstract—The interaction of the monoamine oxidase inhibitor pargyline with cultured hepatocytes has been studied. [Phenyl-3, benzyl-^{3}H] pargyline (38 nM) rapidly enters the cells and a plateau of incorporation into a trichloroacetic acid insoluble form (monoamine oxidase) is reached after 2 hr. The level of labelling is lower in freshly isolated cells than in those in later culture. The maximum incorporation accounts for only 6% of the added radioactivity and produces a 9% inhibition of monoamine oxidase activity. The remaining [^{3}H] pargyline is metabolized and quickly accumulates in the cell culture medium in a form which cannot label exogenous mitochondria. The metabolism of pargyline varies both qualitatively and quantitatively with culture age. In 0 hr and 20 hr-cultured cells one metabolite preferentially appears whilst in 140 hr cultured hepatocytes at least three metabolites are formed. The metabolism of [^{3}H] pargyline in early culture is consistent with a cytochrome P-450 involvement. The use of [^{3}H] pargyline to label monoamine oxidase in cultured hepatocytes offers several attractive features for studying the turnover of this enzyme. These include speed of interaction, non-reutilization, application to normal cells, controlled inhibition of monoamine oxidase and metabolism of non-specific label.

The mechanism(s) involved in intracellular protein degradation are poorly understood [1, 2]. Problems using radioisotopes to monitor the degradation of defined proteins include the lack of specificity of the label and its eventual reutilization following the breakdown of the original tagged protein.

In a previous study we have shown that the enzyme monoamine oxidase (MAO:EC 1.4.3.4), in cultured hepatocytes, can be specifically labelled with low concentrations of its irreversible inhibitor [^{3}H] pargyline [3]. Cells were later incubated with a high concentration of cold pargyline and the degradation of the enzyme was subsequently recorded. Under these conditions MAO was totally inhibited. Although MAO inhibitors are widely used clinically knowledge of their pharmacokinetics is limited [4]. We are currently using [^{3}H] pargyline labelled MAO as a probe in investigations of the effect of long term culture on mitochondria. A steady state or a positive nitrogen balance in cultured hepatocytes is dependent on the use of a culture medium containing high concentrations of amino acids [5, 6]. Liver tissue is very rich in aromatic amino acid decarboxylases [7, 8] and continuous inhibition of MAO by pargyline would be expected to generate a range of amines in hepatocytes. Serotonin and adrenaline inhibit protein degradation in skeletal muscle *in vitro* [9, 10] and the generation of other lysosomotropic amines [11] would also impair protein degradation.

In view of these facts the present investigation was designed to study the utilization of pargyline by cultured hepatocytes. The uptake of radioactive pargyline by MAO was determined and the nature of the free radioactivity was also investigated. The effect of culture duration on the fate of pargyline was determined by using hepatocyte cultures of different ages.

MATERIALS AND METHODS

Chemicals. Collagenase was supplied by Boehringer Mannheim, insulin from Boots, Nottingham, U.K. and Opti Phase X from LKB, Croydon, Surrey, U.K. Leibovitz L-15 culture medium, newborn calf serum and gentamycin were obtained from Flow Laboratories, Irving (Ayrshire, Scotland, U.K.). [^{14}C]Tyramine (56 mCi/mmol) was from Amersham International (Amersham, U.K.) and [phenyl-3-benzyl-^{3}H] pargyline hydrochloride (20.5 Ci/mmol) from New England Nuclear Corp. (Dreieich, Germany). Pargyline hydrochloride was obtained from two sources, namely from Abbott Laboratories, (Chicago, U.S.A.) and from Sigma, Poole, Dorset, U.K. All other chemicals were obtained from Sigma.

Preparation and culture of hepatocytes. Hepatocytes were prepared as described previously [12, 5]. Cells were cultured on Primaria culture dishes (60 mm Falcon). The culture medium was Leibovitz L-15 medium pH 7.4 supplemented with glucose (8.3 mM), Hepes (25 mM), gentamycin (50 μg/ml), insulin (0.8 μg/ml), dexamethasone (1 μM) and 10% (v/v) heat inactivated (56° for 30 min) newborn calf serum. Hepatocytes (2.5×10^6) were plated out in 3 ml of the culture medium. The medium was changed after 2.5 hr and subsequently at 24 hr intervals unless otherwise indicated.

* To whom correspondence should be sent.

Labelling of endogenous hepatocyte monoamine oxidase with [^{3}H] pargyline (20.5 Ci/mmol). Hepatocyte culture medium was changed and supplemented with [^{3}H] pargyline (38 nM) at the times indicated (see figure legends). At 30 min intervals the culture medium was removed and frozen at −20° and the cells were harvested.

Cell harvesting. Cells were harvested either by trypsinization [5] or by scraping. Use of Primaria dishes reduced the time needed for trypsin to detach the cells from the dish to under a minute (cf. approx. 20 min for collagen coated dishes [5]). Following trypsinization the cells were washed twice at 4° in 3 ml culture medium, with or without pargyline (100 μM). The cell pellets were either frozen at −20° or resuspended in 1 ml sucrose (0.25 M) and subsequently disrupted and fractionated on Percoll gradients.

Cells were harvested, where indicated, by scraping into 500 μl of sucrose (0.25 M), with or without pargyline (100 μM). Any cells remaining on the plates were scraped into a further 500 μl of the same solution. The cell suspensions were frozen at −20°.

Inhibition of endogenous hepatocyte monoamine oxidase by pargyline. Preliminary experiments revealed that the maximum inhibition of endogenous MAO was obtained after 2 hr. The cell culture medium was changed and supplemented with cold pargyline (0–100 μM). After 2 hr and after 4 hr, 4 plates of each concentration were harvested by trypsinization and all subsequent operations were carried out at 4°. The washed cell pellets were resuspended in 1 ml sucrose (0.25 M) and disrupted by mild sonication [13] (one 10 sec burst at an amplitude setting of 4 μm peak to peak with an MSE Soniprep 150). The mitochondrial fraction was separated from the cytosol by Percoll gradient centrifugation. Fractionated gradients were assayed for lactate dehydrogenase (EC 1.1.1.27), MAO and succinate dehydrogenase (EC 1.3.99.1). The presence of lactate dehydrogenase in the top Percoll fractions demonstrated that intact cells had been effectively disrupted. The inhibition of MAO by a given pargyline concentration was determined by reference to the appropriate control gradient obtained from cells cultured in the absence of pargyline. Total cell numbers applied to different gradients were standardized by correcting for the total mitochondrial marker enzyme succinate dehydrogenase.

Incubation of Percoll gradient fractions from pargyline treated cells with exogenous MAO (see later) for the duration of the MAO assay caused no inhibition of activity. Any pargyline remaining initially in the cells had therefore been effectively diluted and the method used measures only the inhibition produced by pargyline in the intact cells during culture.

Percoll gradient centrifugation. One ml of the broken cell preparations were layered on 7.0 ml of Percoll (30%, v/v) in iso-osmotic sucrose (0.25 M) in 10 ml centrifuge tubes. Centrifugation was for 25 min at 53,000 *g* in an MSE Superspeed 65 centrifuge. Gradient fractions (0.5 ml) were collected and frozen (−20°).

Thin-layer chromatography-analysis of soluble radioactivity. This was carried out on aluminium sheets (20 × 20 cm) precoated with a 0.2 mm thick layer of silica gel (60 F_{254} Merck, Darmstadt, Germany). Culture media from [^{3}H] pargyline cultured cells were used. Interfering protein was removed by adding trichloroacetic acid (10% w/v final concentration) and leaving the preparation in ice for 30 min. A total of 10 μl of the resulting supernatants were applied, spotwise, to the thin-layer plates. The spots were dried in a stream of air between each application. Chromatography was carried out either in butanol:glacial acetic acid:water (4/1/2 by vol.) or in chloroform:methanol:formic acid (85/15/1 by vol.). Following chromatography the plates were allowed to dry in air. The position of pargyline on the plate was determined by the mobility of a standard pargyline preparation (0.1 μmol). The latter was localized in ultraviolet light from a mineral light UVS L-25. The distribution of radioactivity on the plates was determined by cutting the plate into 0.5 cm sections. The radioactivity was measured using Opti Phase X as scintillant with a LKB 1219 Rackbeta liquid scintillation counter. The recovery of radioactivity from the plates was 85 ± 18% (N = 64).

Preparation and labelling of hepatocyte mitochondria by [^{3}H] pargyline medium conditioned by hepatocytes. A mitochondrial preparation was obtained from hepatocytes (1.3×10^8 in 0.25 M sucrose). The hepatocytes were sonicated (1 × 10 sec burst amplitude setting 4.5 μm peak to peak) and centrifuged at 500 *g* for 10 min. The supernatant was retained and centrifuged at 500 *g* for a further 10 min. The 500 *g* supernatant was then centrifuged at 7500 *g* for 10 min. The resulting pellet, containing 60% of the initial MAO activity, was used for the labelling studies. The pellet was resuspended in 2 ml sucrose (0.25 M) and the MAO activity was 0.79 n mol/min/mg protein.

A 250 μl portion of the mitochondrial preparation was added to 1.5 ml of cell culture medium from the [^{3}H] pargyline labelled cells (for further details see figure legends). The preparation was incubated at 37° with gentle shaking. At 30 min intervals samples were withdrawn and analysed for trichloroacetic acid insoluble label and MAO activity.

Analyses. Protein and the activity of lactate dehydrogenase were determined as described previously [12]. The activities of the mitochondrial enzymes succinate dehydrogenase and MAO were determined by the methods of Pennington [14] and Russell and Mayer [15] respectively. Cytochrome P-450 was determined by the method of Omura and Sato [16]. Unbroken hepatocytes pellets were thawed and resuspended in 1 ml 0.1 M potassium phosphate buffer pH 7.6. The cells were broken by 2 × 10 sec burst at an amplitude setting of 6 μm peak to peak in an MSE soniprep 150. The containing tube was cooled in ice. The radioactive [^{3}H] associated with protein was determined after precipitation with trichloroacetic acid (10% w/v final concentration) containing pargyline (1 mM). The precipitates were redissolved and reprecipitated. The precipitates were then dissolved in formic acid (50% v/v) and the radioactivity was measured with Opti Phase X as scintillant.

RESULTS AND DISCUSSION

Cultured hepatocytes rapidly incorporate [^{3}H] pargyline into a TCA insoluble form which reaches a maximum value after 2 hr (Fig. 1). Previously we have shown this form is pargyline labelled MAO [3]. The labelling has no affect on the viability of the cells as judged by stable lactate dehydrogenase and protein measurements. Residual, cellular unbound pargyline, following the labelling period, does not appear to be a problem since the presence of cold pargyline (100 μM) during cell harvesting does not reduce the counts incorporated. This demonstrates that unbound radioactive pargyline does not bind after cell harvesting. Similarly, Percoll fractionation of labelled cells shows that following the washing procedure, only background radioactivity is present in the cell cytosol. Moreover, pargyline concentrations of 100 μM are effectively removed from the cells. This is demonstrated by the inability of Percoll gradient fractions of cells cultured at this concentration of pargyline to inhibit exogenous MAO. All of the radioactivity on the gradients is TCA insoluble and coincident with the mitochondrial enzymes MAO and succinate dehydrogenase. The presence of cold pargyline (100 μM) during the labelling period reduces the gradient counts to zero.

The labelling of MAO (±SD) after 0, 20 and 140 hr culture is 13, 731 ± 2691 (N = 3), 29, 082 ± 9897 (N = 5) and 25, 422 ± 13,635 (N = 3) dpm/mg protein respectively. These values are for quadruple plates where N is the number of separate rat liver preparations. The variation between plates from the same animal was always less than 9%. Variation between different animal livers increased with culture age but for the same animal liver the labelling of the 0 hr cultures was consistently lower than the labelling achieved in older cultures.

The concentration dependence of the inhibition of MAO by pargyline in cultured hepatocytes is shown in Fig. 2. Both the [^{3}H] and non-radioactive pargyline at 38 nM give an inhibition of 9% showing that they behave in the same manner. The maximum association of the [^{3}H] pargyline label with cellular protein is only 6%, the remaining [^{3}H] (94%-[^{3}H] pargyline equivalent 35.7 nM) is in a TCA soluble form (10% w/v final concentration) in the cell culture medium. A pargyline concentration of 35.7 nM does not appear to represent a threshold value for entry into the cell since, as shown in Fig. 1, a second later dose of [^{3}H] pargyline results in only approximately a doubling of the label incorporated into MAO. The inhibition of MAO by different pargyline concentrations in cultured hepatocytes (Fig. 2) is similar to that produced by the inhibitor in homogenate systems [17]. This emphasizes that the hepatocyte membrane is not a barrier to the passage of pargyline into the cell. The direct proportionality between MAO inhibition and pargyline concentration (Fig. 2) suggests that all mitochondria within the cell are

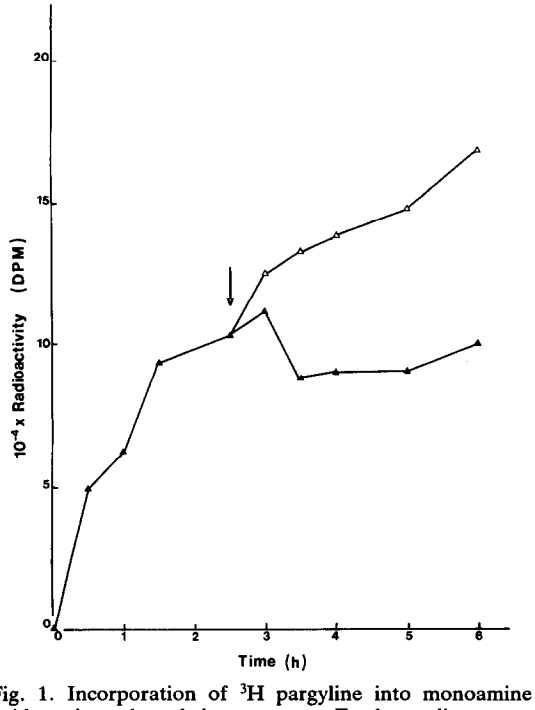

Fig. 1. Incorporation of ^{3}H pargyline into monoamine oxidase in cultured hepatocytes. Fresh medium supplemented with [^{3}H] pargyline (38 nM) was added to a hepatocyte culture after 40 hr incubation and at the time indicated by the arrow a second dose of the same concentration was added. Cells were harvested by scraping. The protein content of the monolayer was 4.2 mg/plate. ▲ Cellular trichloroacetic acid insoluble radioactivity (single dose [^{3}H] pargyline) △ cellular trichloroacetic acid insoluble radioactivity (double dose [^{3}H] pargyline). Values are the mean of duplicate plates.

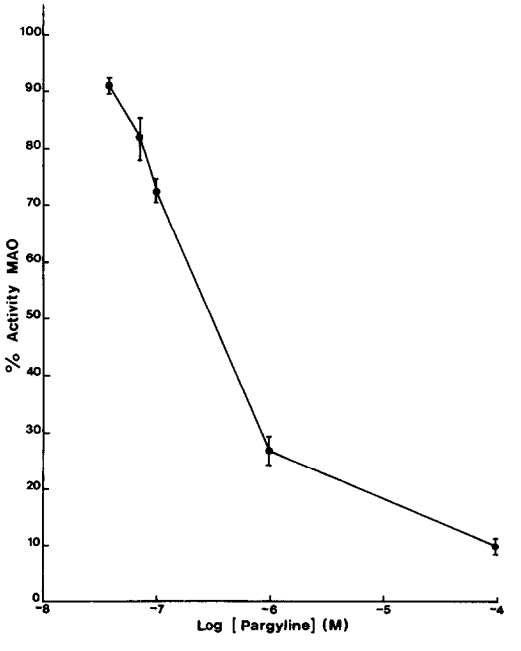

Fig. 2. Dose-dependent inhibition of monoamine oxidase by pargyline in cultured hepatocytes. Hepatocyte cultures of 24 hr were used. The monoamine oxidase activity layered onto the gradients was 0.15 nmol/min/mg protein. The values are the mean ± SD (N = 3) except at the lowest dose of pargyline tested where N = 5.

equally accessible to labelling. An alternative explanation, valid if the [^{3}H] remained throughout as pargyline, would be that mitochondria are progressively harder to label.

Analysis of the radioactivity in the cell culture medium was undertaken by thin layer chromatography. In the solvent system of butanol: acetic acid: water the [^{3}H] pargyline moves as a single peak with an R_f of 0.60. This is coincident with cold pargyline which was localized in ultraviolet light. Chromatography in chloroform: methanol: formic acid separates the cold pargyline (both Sigma and Abbott) into two ultraviolet spots of R_f 0.60 and 0.75 The spot of R_f 0.75 contains 96% of the total radioactivity and was taken to be pargyline. The second ultraviolet spot is not radioactive and is an impurity in the cold pargyline seen due to the high concentration applied to the plate. A second small radioactive peak is located with R_f of 0.4 (Fig. 4a). There is no corresponding spot visible for the reference pargyline under ultraviolet light. The chloroform: methanol: formic acid system is clearly the more effective in resolving "pargyline like" components and was used in all the subsequent investigations.

Protein precipitation by TCA (10% w/v final concentration) causes no change in the mobility of pargyline. Similarly incubation for 6 hr in complete cell culture medium has no effect on [^{3}H] pargyline (Fig. 4a). Changes in the behaviour of the radioactivity following exposure to hepatocytes are therefore attributable to cell-mediated events rather than to any inherent instability of [^{3}H] pargyline under the conditions used. Figure 3(a–h) shows that in 20 hr cultured hepatocytes the [^{3}H] pargyline peak (1) is rapidly depleted and replaced by a new radioactive peak (2) with an R_f of 0.56. This pattern is also observed in 0 hr cultured hepatocytes. In 140 hr cul-

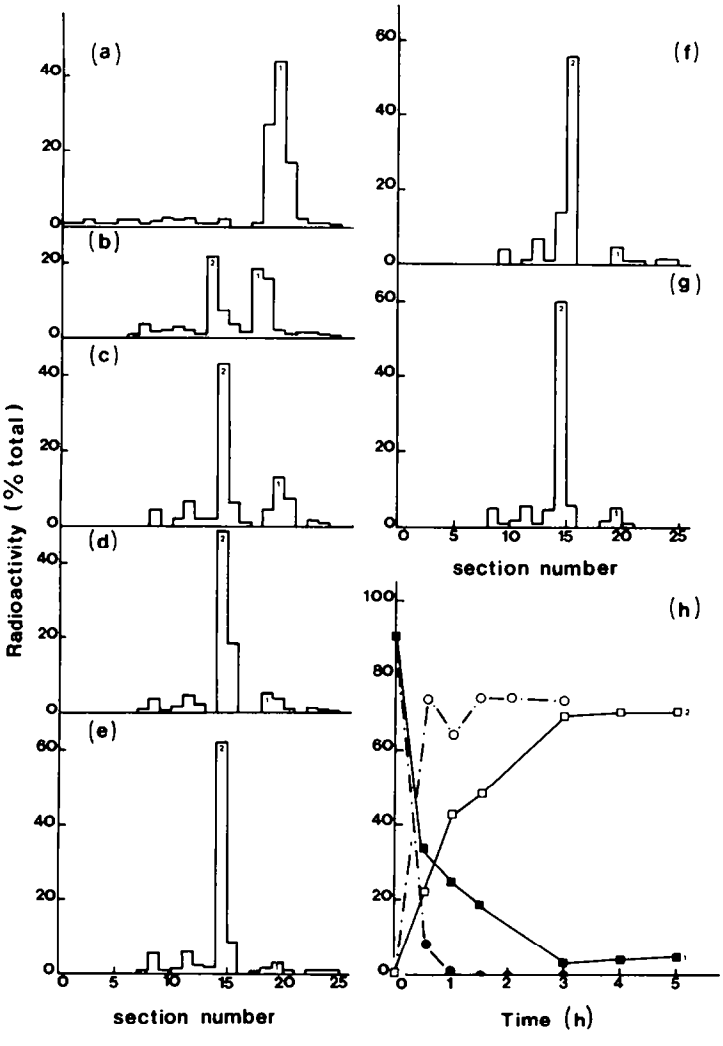

Fig. 3. Thin-layer chromatographic analysis of cell culture medium from 20 hr cultured hepatocytes incubated with [^{3}H] pargyline. (a–g) Show the distribution of radioactivity at 0, 0.5, 1, 1.5, 3, 4 and 5 hr respectively. The sections (0.5 cm) are numbered from the origin and the radioactivity applied per run was 7000 dpm. (h) Shows the reduction in the pargyline peak (1) ■ and the appearance of the major metabolite (2) □ as a function of time. ●·—·● and ○—·—○ are the corresponding values from 0 hr cultured hepatocytes.

tured hepatocytes the situation is more complex (Fig. 4). Three new radioactive peaks appear (3, 4 and 5: R_f's 0.48, 0.39 and 0.28 respectively) as the pargyline peak declines. The rate of pargyline utilization decreases with the age of the culture (Fig. 3h and 4h). Hepatocytes of all ages convert [^{3}H] pargyline to other forms which accumulate in the medium. It is apparent that [^{3}H] pargyline may travel along at least 5 separate and mutually exclusive pathways in cultured hepatocytes. Firstly, it may be incorporated into the enzyme MAO. Secondly, in 0 hr and 20 hr cultured cells, it may be preferentially converted to a single alternative form. In 140 hr cultures the third, fourth and fifth alternatives are dominant whereby pargyline undergoes transition to three new forms which are generated by distinct mechanisms since there is no interconversion between the peaks (Fig. 4b–h). In 20 hr cultured hepatocytes (Fig. 3a–g) small peaks progressively appear with lower R_f's than the major metabolite. These minor peaks are not seen in 0 hr cultured hepatocytes and have identical R_f's to those of the three major metabolites seen in 140 hr cultured hepatocytes. This seems to indicate that the mechanisms for dealing with pargyline at 140 hr are at least present at 20 hr but their effects are minimal due to the dominant mechanism present in the cells earlier in culture. The dominant mechanism is not apparent in 140 hr cultures. The thin layer chromatographic analyses of the [^{3}H] in the cell culture medium does not support the proposal that the mitochondria are progressively harder to label since the [^{3}H] pargyline is being rapidly converted to other forms in cultured hepatocytes.

All the metabolites of [^{3}H] pargyline produced by cultured hepatocytes appear outside the cells. Figure 5 shows the ability of these forms to label exogenous mitochondria. Under the incubation conditions, preliminary experiments showed that MAO activity is

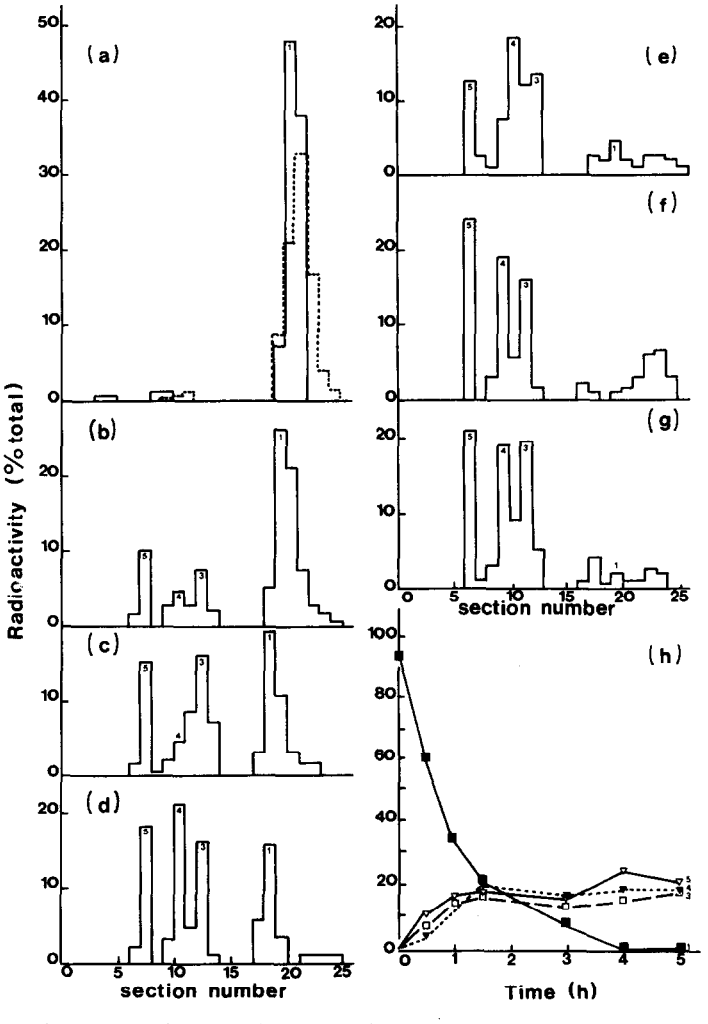

Fig. 4. Thin-layer chromatographic analysis of cell culture medium from 140 hr cultured hepatocytes incubated with [^{3}H] pargyline. (a) Shows the distribution of stock [^{3}H] pargyline (---) and the solid line shows the distribution of pargyline which has been incubated in cell culture medium for 5 hr. (b–g) Shows the distribution of radioactivity at 0.5, 1, 1.5, 3, 4 and 5 hr respectively. The sections (0.5 cm) are numbered from the origin and the radioactivity applied per run was 7000 dpm except (a) (---) where 100,000 dpm was applied. (h) Shows the reduction in the pargyline peak (1) ■ and the appearance of the three major metabolites 3 (□) 4 (▼) and 5 (▽) as a function of time.

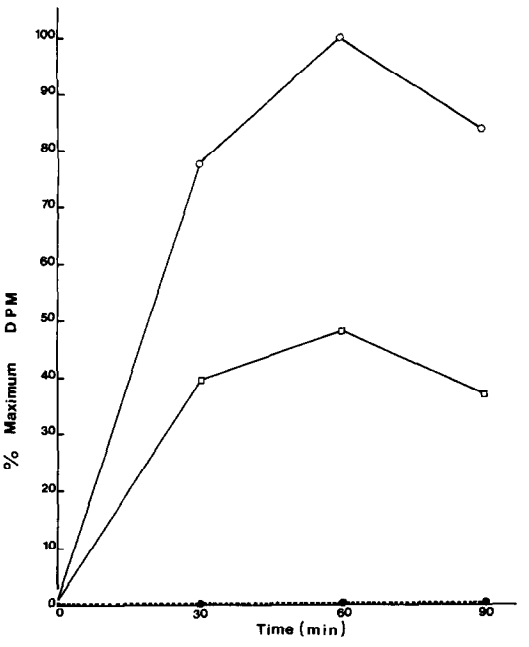

Fig. 5. Labelling of hepatocyte mitochondria by [^{3}H] pargyline medium conditioned by hepatocytes. ○, TCA insoluble label incorporated into mitochondria in sucrose (0.25 M) containing [^{3}H] pargyline; □, TCA insoluble label incorporated into mitochondria in cell culture medium containing [^{3}H] pargyline which had been immediately removed from hepatocyte cultures; ●---●, TCA insoluble label incorporated into mitochondria in cell culture medium initially containing pargyline which had been incubated with either 20 hr or 140 hr cultured hepatocytes for 5 hr. The concentration of [^{3}H] pargyline in all cases was 32.5 nM (refer to Materials and Methods section). Values are the mean of duplicate results.

stable for 30 min and then falls linearly. However, at least 65% of the activity remains after 90 min so that the inability of [^{3}H] to label MAO cannot be attributed to enzyme denaturation. Medium containing [^{3}H] pargyline, immediately removed from hepatocyte cultures, is capable of labelling exogenous mitochondria. The complex medium containing serum impairs the ability of [^{3}H] pargyline to label MAO reducing it to 48% of the labelling which is achieved in sucrose (0.25 M) containing [^{3}H] pargyline, but unchanged [^{3}H] pargyline is still clearly able to label MAO. Nevertheless, cell culture medium containing [^{3}H] pargyline exposed to either 20 hr or 140 hr cultured hepatocytes for 5 hr is unable to label exogenous mitochondria (maximum 0.4% of sucrose value). Hence the cells have not merely converted the [^{3}H] pargyline into forms which cannot accumulate in the cell but the transformation of the [^{3}H] leads to forms which can no longer label MAO.

The [^{3}H] pargyline labelling of MAO in cultured hepatocytes has many attractive features for use in studying the turnover of this enzyme. The label is specific and, due to the nature of its covalent binding to MAO, cannot be reutilized to label new MAO molecules [18, 19]. Pargyline readily enters through the hepatocyte membrane and can be rapidly removed from the cells. The labelling is rapid (Fig. 1) and due to the abundance of the enzyme in liver cells, is also considerable. This allows degradation measurements to be accurately made for the extended periods required for an enzyme which does not turn over rapidly. In comparison the [^{14}C] biotin labelling of pyruvate carboxylase in 3T3 fibroblasts requires a 48 hr period in the presence of inducers whilst the cells are converted to adipocytes [20]. At 38 nM only 9% of the total MAO activity is inhibited ensuring that complications due to the accumulation of amines does not occur even in long-term cultures. The rapid metabolism of pargyline would appear to have two immediate consequences. First cold pargyline is not required to prevent new labelling after the initial 2 hr and second the metabolism increases the specificity of labelling by reducing the concentration of pargyline to very low levels ensuring only sites with a high affinity are labelled (cf. nonspecific binding of pargyline to rat hepatoma mitochondria in a cell-free system [21]).

We are currently investigating the nature of the difference in the metabolism of pargyline by 20 hr and 140 hr hepatocytes. Recently Yoshida *et al.* [22] reported that the related MAO inhibitor deprenyl is metabolized *in vivo* by the cytochrome P-450 system. Cytochrome P-450 is known to decline rapidly during the first 24 hr of culture and then to fall at a slower rate [23]. Our results are consistent with a cytochrome P-450 involvement early in culture which is later replaced by several slightly slower mechanisms. In 0 hr cultured hepatocytes cytochrome P-450 levels are 0.93 ± 0.15 nmol/mg protein ($N = 4$) which subsequently fall to 0.54 ± 0.14 nmol/mg protein ($N = 3$) over the 20 hr period and, under the culture conditions used have fallen to 0.11 ± 0.05 nmol/mg protein ($N = 3$) after 140 hr. A cytochrome P-450 involvement is consistent with the lower amount of labelling of MAO after 0 hr culture as opposed to later times. This lower level of labelling is not due to the atypical mitochondrial configuration following perfusion damage [24] but, as shown in Fig. 3h and 4h, is caused by a more rapid metabolism of the [^{3}H] pargyline.

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