Biochemical Pharmacology, Vol. 30, No. 11, pp. 1231–1237, 1981.
Printed in Great Britain.

0006-2952/81/111231-07 $02.00/0
Pergamon Press Ltd.

XENOBIOTIC METABOLISM BY ALVEOLAR TYPE II CELLS ISOLATED FROM RABBIT LUNG

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(*Received* 10 *July* 1980; *accepted* 23 *October* 1980)

Abstract—A procedure for the isolation of alveolar type II cells from rabbit lung was developed. Following pulmonary lavage to minimize macrophage contamination, viable cells (30% type II cells) were released by digestion with 0.1% Protease type I (Sigma) in 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES)-buffered balanced salt solution containing 0.5 mM ethyleneglycolbis (amino-ethylether) tetra-acetate (EGTA) instilled via the trachea. Type II cells were enriched to 50–60 per cent purity by centrifugal elutriation. Density gradient centrifugation in metrizamide was used to increase the purity of the type II cells from the elutriator fraction to 80 per cent. Whole, freshly isolated alveolar type II cells metabolized 7-ethoxycoumarin at a rate of 30 pmoles umbelliferone formed per mg protein/min. However, only traces of coumarin hydroxylase activity were detected which could be accounted for by 1–2% Clara cell contamination in the type II cell fraction. NADPH–cytochrome *c* (cyt *c*) reductase activity in the sonicated type II cell fraction was 44 nmoles cyt *c* reduced per mg protein/min compared to 25 for lung homogenate. Benzo[*a*]pyrene hydroxylase and *N*,*N*-dimethylaniline *N*-oxidase activities were also demonstrated in the type II cell fraction to be 13 pmoles 3-OH benzo[*a*]pyrene · (mg protein)$^{-1}$ · min^{-1} and 0.8 nmole DMA *N*-oxide · (mg protein)$^{-1}$ · min^{-1} respectively. Microsomes prepared from the isolated type II cell fraction contained 74 pmoles cytochrome P-450/mg protein and 90 pmoles cytochrome b_5/mg protein.

Because of the increasing incidence of lung disease associated with environmental factors and chemicals, study of xenobiotic metabolism in isolated pulmonary cells is now of considerable interest. Many chemicals require activation by enzymes of the mixed-function oxidase (MFO) system to exert their toxicity in various tissues. The localization of these toxication–detoxication reactions in distinct bronchoalveolar cells or regions may be related to the location of pulmonary damage and disease in certain cell types [1]. Because of the complexity and heterogeneity of the lung (about forty cell types [2]), however, individual cell populations have been unavailable for this kind of study in the past.

Since Kikkawa and Yoneda [3] first isolated the alveolar type II cell less than ten years ago, numerous investigations have been undertaken to study the functions of this cell type. Alveolar type II cells, which are recognized by their characteristic lamellar inclusion bodies, have been demonstrated to secrete pulmonary surfactant into the alveolar space [4, 5]. A second role attributed to the type II cell is that of a progenitor for the type I cell both during normal development [6] and after type I cell injury [7, 8]. Other functions have been postulated for the type II cell, but they have not yet been demonstrated.

Recently, our laboratory has begun to study foreign compound metabolism in isolated pulmonary cells [9]. Using mixed populations of cells, we have demonstrated that the alveolar type II cell exhibits some MFO activity. The type II cell, however, does not seem to be involved in all xenobiotic biotransformations that occur in the lung.

In this report, we have studied the ability of isolated rabbit alveolar type II cells to metabolize 7-ethoxycoumarin (7-Ec), benzo[*a*]pyrene, and other substrates. We have also related these activities to those found in the whole lung.

MATERIALS AND METHODS

4-(2-Hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES)-buffered balanced salt solution (HpBS) used for cell isolation and Krebs Ringer–BSA perfusion medium have been described previously [9]. Metrizamide was obtained from the Accurate Chemical & Scientific Corp., Hicksville, NY. Protease type I was purchased from the Sigma Chemical Co., St. Louis, MO. Phosphine 3R dye was obtained from the Roboz Surgical Instrument Co., Washington, DC.

Lungs from male New Zealand white rabbits (Dutchland Animals, Denver, PA) weighing 2–3 kg were used for all cell preparations. The perfusion and cell preparation procedures have been described previously [9, 10].

Digestion of lung tissue with 0.1% Protease type I and 0.5 mM ethyleneglycolbis (amino-ethylether) tetra-acetate (EGTA) [10] yielded a cell digest that contained 30% alveolar type II cells. Initial separation of these cells was made by centrifugal elutriation [9]. Four fractions were collected on the basis of size, the smallest cells and particles eluting first. Elutriator fraction 2, which contained 50–60% type

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II cells, was collected at a centrifuge speed of 2000 rpm and a flow rate of 15 ml/min. Elutriator fraction 4, which was collected at a centrifuge speed of 1200 rpm and a flow rate of 28 ml/min, contained 30% Clara cells and only about 10% type II cells. Further separation of the type II cell population in elutriator fraction 2 was made by metrizamide density gradient centrifugation [11]. This cell fraction (8×10^7 cells in 5 ml) was layered onto a 25-ml solution of 12.5% metrizamide in HpBS which was then centrifuged in a Beckman SW 27 rotor at 3000 *g* for 1 hr at 4°. The type II cells were enriched in the top band at the 1.06 density interface.

Cell counts were made using a hemacytometer. Viability of cells was estimated by the trypan blue (0.04%) dye exclusion method [12]. Since NADPH does not cross intact cell membranes, MFO activities in freshly isolated cells in the presence and absence of added NADPH were compared, as another indicator of cell viability. Alveolar type II cell identification and enumeration were made using phosphin 3R [9, 13] and the modified Papanicolou (PAP) stain without acid alcohol [3, 10].

For electron microscopy, pellets of alveolar type II cells were fixed and processed by the method of Williams [14]. Cell preparations were examined in a Phillips model 300 electron microscope.

All assays were performed with freshly isolated cells. The amount of protein in the cellular, homogenate, and microsomal fractions was measured by the method of Lowry *et al.* [15]. Enzyme activity data were expressed on a per mg protein basis to compare activities in cell fractions with lung homogenates and with cell digest that contained much debris. The M-1 fraction (type II cells) contained approximately 8×10^6 cells/mg cellular protein.

7-Ec deethylase and coumarin hydroxylase activities were assayed by fluorimetric measurement of umbelliferone production. A modification of the method of Ullrich and Weber [16] was used. Whole or sonicated cells and homogenate and microsomal fractions in HpBS were incubated with 0.3 mM 7-Ec (except where noted) or 1.6 mM coumarin in the presence or absence of 1 mM NADPH for 15 min at 37°. When acetone was used as the vehicle for 7-Ec (4 μl in 1 ml of reaction mixture), it had no effect on the reaction. The reactions were stopped with trichloroacetic acid, the protein was removed by centrifugation, and the supernatant fractions were made basic with Tris–glycine buffer, pH 9.0. Following this treatment, a step was included to extract 7-Ec with heptane (not needed for coumarin incubations). Fluorescence of the umbelliferone was measured at an excitation wavelength of 375 nm and an emission wavelength of 458 nm.

NADPH–cytochrome *c* reductase was measured in sonicated cells by the method of Peters and Fouts [17] on a Gilford 2400 recording spectrophotometer. The reduction of cytochrome *c* was followed at 550 nm using an extinction coefficient of 18.5 $cm^{-1}mM^{-1}$. The reaction mixtures contained 60 mM KCl and were incubated at 30°.

Benzpyrene hydroxylase was measured by the micromethod of Sweeney *et al.* [18], which is a modification of the method of Poland and Glover [19]. An Aminco–Bowman spectrofluorometer was used for measuring the fluorescence. It was standardized with quinine sulfate which, in turn, was standardized against 3-hydroxy-benzo[*a*]pyrene. The excitation and emission wavelengths were 388 and 520 nm respectively.

Measurement of *N,N*-dimethylaniline (DMA) *N*-oxidase activity has been described previously [20, 21]. Sonicated cells were incubated with an NADPH-generating system and 4 mM DMA at 37° for 15 min at pH 8.9 (pH optimum for this enzyme in pulmonary microsomes [21]).

Cytochrome P-450 was quantified from the CO-difference spectra of dithionite-reduced microsomes between the peak wavelength (near 450) and 490 nm [22]. An extinction coefficient of 91 $cm^{-1}mM^{-1}$ for cytochrome P-450 was used. The NADH difference spectra of microsomal suspensions at 409–424 nm were measured to determine cytochrome b_5 using an extinction coefficient of 185 $cm^{-1}mM^{-1}$ [22]. Microsomal pellets for these cytochrome determinations were prepared by centrifugation (100,000 *g* for 25 min) of the 9000 *g* supernatant fraction from sonicated cell fractions.

RESULTS

Alveolar type II cells were isolated from rabbit lung to 80 per cent average purity (see Materials and Methods). This cell fraction also contained about 2% Clara cells and 7% macrophages, but no other cell types were present in amounts greater than 2%. The yield of type II cells from four rabbits was $1.0 \times 10^8 \pm 0.5 \times 10^8$ cells (mean ± S.D., N = 14). These cells contained the characteristic lamellar bodies (Fig. 1) that have been observed in type II cells *in situ* [23]. All other intracellular organelles appeared intact as well. Trypan blue dye exclusion consistently gave viability estimates of about 90 per cent or greater for these isolated cells. 7-Ec deethylase activity in alveolar type II cells in the presence or absence of NADPH, however, indicated lower viability when compared to the dye exclusion method. 7-Ec deethylase activity in type II cells in the absence of NADPH was only about 70 per cent of the activity in the presence of NADPH. Therefore, type II cells were incubated in HpBS for 10 min at 37° and then their viability was remeasured by dye exclusion. These cells showed only 70 per cent viability, thus demonstrating loss of type II cell viability during incubation. In contrast, viability in the 30 per cent enriched Clara cell fraction was greater than 90 per cent even following 15 min of incubation at 37°. The 80 per cent purified type II cell preparations thus described have been used in all subsequent experiments reported here and are referred to as isolated type II cells or the M-1 fraction.

In characterizing 7-Ec deethylase activity in isolated type II cells, we first examined the effect of protein concentration and cell number on this activity. It was observed that 7-Ec deethylase activity was linear with increasing protein concentration to at least 2.5 mg/ml incubation and with cell number to 2×10^7 cells/ml (data not shown). This activity was also linear with time up to 30 min. Figure 2 shows the effects of varying substrate concentration on the ability of type II cells to deethylate 7-Ec.

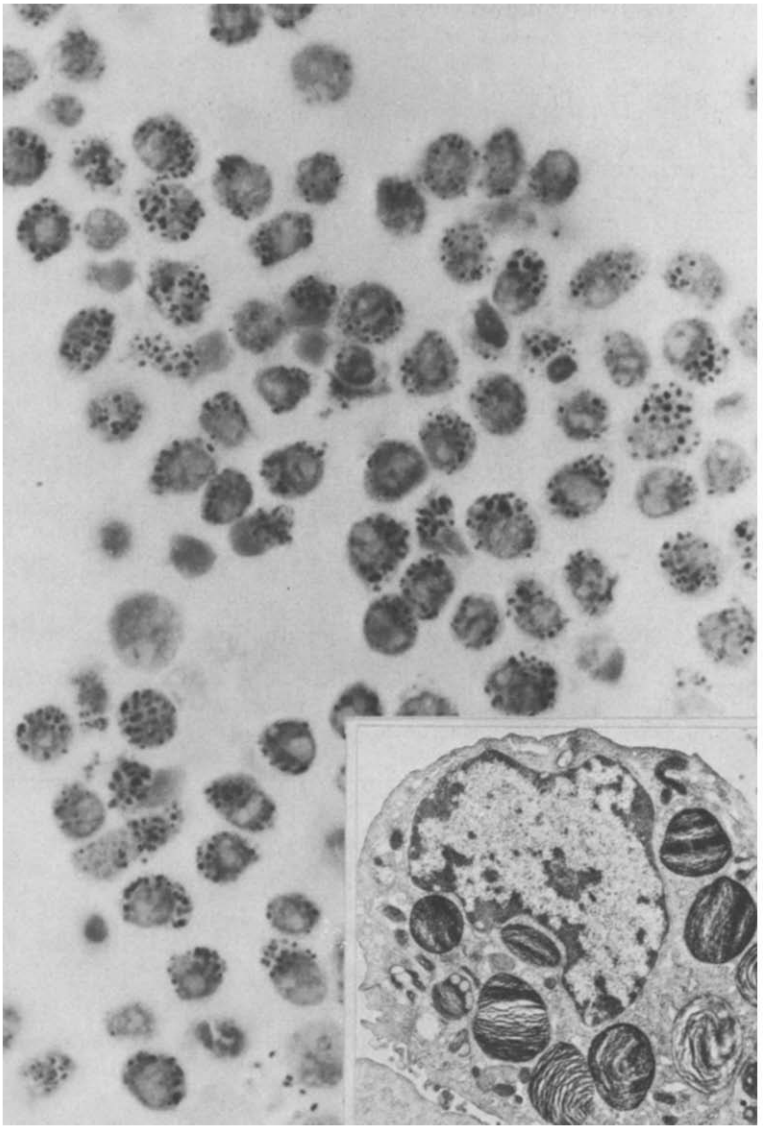

Fig. 1. Modified Papanicolou stain of 80 per cent alveolar type II cell fraction, × 1160. Inset: electron micrograph of alveolar type II cell showing characteristic lamellar bodies, × 6900.

Whereas the saturating 7-Ec concentration for liver microsomes was less than 0.2 mM (data not shown), it was above 0.4 mM for the type II cells. Because of the solubility limit of the substrate in buffer, some of our experiments were performed with 0.4 mM 7-Ec. The maximum rate of reaction, however, could be reached at about 1 mM if acetone was used as the vehicle for 7-Ec. The apparent K_m for 7-Ec deethylation by type II cells was 0.1 mM, compared to 50 μM for the cell digest.

7-Ec deethylase and coumarin hydroxylase activities were measured in alveolar type II cells and compared to the activities in other cell fractions and in whole lung homogenate and microsomes (Table 1). The type II cells were found to have very low coumarin hydroxylase activity, compared with the enriched Clara cell fraction. Coumarin hydroxylase activity seems to be selectively enriched in the Clara cells. In fact, the small amount of coumarin hydroxylase in the type II cell fraction could be accounted for by 2% Clara cell contamination. 7-Ec deethylase activity, however, was present in significant amounts in type II cells. The ratio of 7-Ec deethylase to coumarin hydroxylase activities in the enriched Clara cells was much lower than in lung homogenate and microsomes, indicating that some of the 7-Ec deethylase activity of whole lung must be attributable to cells other than the Clara cell (assuming that the Clara cells account for all measurable coumarin hydroxylase activity). The type II cells have been shown here to exhibit part of this 7-Ec deethylase activity.

Table 2 shows the effects of NADH and SKF-525A, a MFO inhibitor, on 7-Ec deethylase activity in alveolar type II cells. These data were compared to liver microsomes, where NADH has been shown

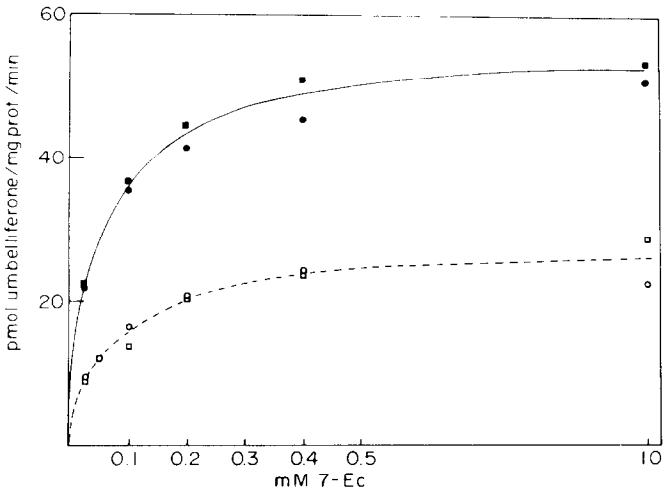

Fig. 2. Effect of [7-Ec] on 7-ethoxycoumarin deethylase activity of alveolar type II cells and cell digest. Key: solid line = cell digest; dashed line = type II cells; ■, □ = whole cells (no NADPH); ●, ○ = sonicated cells plus 1 mM NADPH. Assay conditions are described in Materials and Methods.

Table 1. 7-Ethoxycoumarin deethylase and coumarin hydroxylase activity in cell fractions, lung homogenate, and lung and liver microsomes*

Samples	7-Ec deethylase [pmoles·(mg protein)$^{-1}$·min^{-1}]	Coumarin hydroxylase [pmoles·(mg protein)$^{-1}$·min^{-1}]	Ratio 7-Ec/Coumarin
Cell digest (30% type II, 5% Clara)	49	3.9	7
M-1 (85% type II, 2% Clara)	28	1.4	16
E-4 (49% Clara, 13% type II)	98	26	4
Lung homogenate	117	3.5	33
Lung microsomes	640	26	25
Liver microsomes	1156	158	7

* All samples were sonicated and incubated with 1 mM NADPH. A typical experiment is shown. This experiment was repeated twice with similar results.

Table 2. Effects of cofactors and SKF-525A on 7-ethoxycoumarin deethylase activity in isolated alveolar type II cells compared to liver microsomes*

	Umbelliferone [pmoles/(mg protein/min)]	% Control
Whole cells (WC)	32.5	100
WC + NADPH (1 mM)	42.7	131
WC + NADH (1 mM)	26.1	80
WC + SKF-525A (0.1 mM)	14.4	44
Sonicated cells (SC)	0	—
SC + NADPH	43.2	100
SC + NADH	1.6	4
SC + NADPH + NADH	31.5	73
SC + NADPH + SKF-525A	18.7	43
Lung microsomes (LU) + NADPH	853	100
LU + NADH	74	9
LU + NADPH + NADH	930	109
LU + NADPH + SKF-525A	384	45
Liver microsomes (LM) + NADPH	1008	100
LM + NADH	130	13
LM + NADPH + NADH	1396	138
LM + NADPH + SKF-525A	756	75

* A representative experiment is shown. This experiment was repeated three times with similar results. Assay conditions are described in Materials and Methods.

Table 3. NADPH–cytochrome *c* reductase in M-1 and E-4 cell fractions compared to lung homogenate*

	Cytochrome *c* reduced [nmoles/(mg protein/min)]
M-1 (80% type II)	44 ± 6
E-4 (30% Clara)	76 ± 13
Lung homogenate	26 ± 6

* Each value is the mean ± S.D. (N = 3).

to enhance this enzyme activity, and to lung microsomes. It was demonstrated that NADH could not substitute for NADPH as a cofactor and, in fact, it was shown to be an inhibitor of 7-Ec deethylase in sonicated type II cells. On the other hand, NADH was additive with NADPH for this reaction in lung microsomes and perhaps even synergistic in liver microsomes. SKF-525A was demonstrated to be an inhibitor of 7-Ec deethylase using either whole or sonicated type II cells. This reaction seemed to be even more sensitive to SKF-525A in lung microsomes and type II cells than in liver.

NADPH–cytochrome *c* reductase was also measured in alveolar type II cells and compared to a Clara cell-enriched fraction and to whole lung homogenate (Table 3). There was almost twice as much of this enzyme activity per mg protein in type II cells compared with lung homogenate, indicating some enrichment of this activity in type II cells. The enriched Clara cell fraction showed an even greater concentration of this activity.

Cytochrome P-450 and b_5 were measured in microsomal suspensions from alveolar type II cells, macrophages, cell digests, and whole lung (Table 4). Type II cell microsomes contained 90 pmoles cytochrome b_5/mg protein and 74 pmoles cytochrome P-450/mg protein. The average concentrations of cytochrome P-450 in cell digests were about the same as in lung homogenates, although levels of cytochrome P-450 in cell digests were more variable between experiments; cytochrome b_5 was enriched in the cell fractions compared to lung homogenate.

Other xenobiotic metabolism enzyme activities found to be present in alveolar type II cells included benzo[*a*]pyrene hydroxylase and DMA *N*-oxidase (Table 5). Although these activities were highest in the enriched Clara cell fractions, the type II cells exhibited both activities to about the same extent as the cell digest on a per mg protein basis.

To see if Protease I had any differential effect on lung cells or on metabolism by these cells, 7-Ec deethylase was measured in whole alveolar type II cells and in a 60% enriched fraction of Clara cells in the presence or absence of 0.001, 0.01 or 0.1% Protease I (incubation time, 15 min). We found that there was less than a 20 per cent decrease in 7-Ec deethylase activity with any of these concentrations of Protease I in either of the whole cell fractions, indicating that the protease was not preferentially destroying one of these cell types. However, 0.01% Protease I destroyed 90 per cent of the 7-Ec deethylase activity in lung homogenate, indicating that this enzyme disrupts MFO activity if it enters the cells.

Since the cell digest contained a large amount of debris, we also wanted to see if any residual protease activity was present which might decrease xenobiotic metabolism activity in this fraction during cold storage (while the purified cell fractions were being prepared). 7-Ec deethylase and coumarin hydroxylase were measured in freshly prepared cell digests and in cell digests stored on ice for 4 hr as it

Table 4. Cytochrome b_5 and P-450 contents in microsomal suspensions prepared from cell fractions and whole lung homogenate*

	Cytochrome b_5 (nmoles/mg protein)	Cytochrome P-450 (nmoles/mg protein)
Cell digest	0.125 ± 0.028	0.152 ± 0.074
M-1 (80% type II cells)†	0.090 ± 0.005	0.074 ± 0.036
Whole lung	0.039 ± 0.012	0.146 ± 0.015
Macrophages	0	0

* Each value is the mean ± S.D. (N = 3).

† 2×10^8 Cells yielded approximately 2 mg microsomal protein (see Materials and Methods).

Table 5. Benzpyrene hydroxylase and *N,N*-dimethylaniline *N*-oxidase activities in cell digest, type II, and enriched Clara cell fractions*

	Benzpyrene hydroxylase [pmoles·(mg protein)$^{-1}$·min^{-1}]	DMA *N*-oxidase [nmoles·(mg protein)$^{-1}$·min^{-1}]
Cell digest	16	1.0
M-1 (80% type II, 2% Clara cells)	13	0.8
E-4 (30% Clara, 13% type II cells)	44	1.7

* A representative experiment is shown. This experiment was repeated two times with similar results. Incubation and assay conditions are described in Materials and Methods.

was usually prepared for assays. The cells were sonicated just prior to incubation, and 1 mM NADPH was added. Coumarin hydroxylase activity was low but unchanged in both preparations while 7-Ec deethylase activity decreased 40 per cent [from 28 pmoles·min^{-1}·(mg protein)$^{-1}$ to 17 pmoles·min^{-1}(mg protein)$^{-1}$]. 7-Ec deethylase activity in whole cells (no NADPH added) from cell digest did not decrease with time. This indicates that there may have been small amounts of residual protease in the cell digest preparations which caused part of the difference between the activities seen in the cell digest and the lung homogenate in Table 1, as well as the variation in cell digest activities between experiments. Protease action on the cell debris and broken cells in the cell digest during tissue digestion may also account for much of the difference in enzyme activities between cell digest and whole lung homogenate.

DISCUSSION

In this report we have demonstrated that alveolar type II cells isolated from rabbit lung exhibit a significant amount of pulmonary xenobiotic metabolism as measured by 7-Ec deethylase, benzpyrene hydroxylase, and DMA *N*-oxidase activities. This type II cell fraction, however, displayed only a trace amount of coumarin hydroxylase activity, which might be accounted for by Clara cell contamination. Coumarin hydroxylase activity seemed to be enriched specifically in the Clara cell fractions. This indicates that there may be qualitative as well as quantitative differences between the ways type II and Clara cells metabolize xenobiotics.

Serabjit-Singh *et al.* [24] have shown that an antibody to cytochrome P-450-I inhibits 100 per cent of rabbit pulmonary microsomal 7-Ec deethylase. In view of the data presented above, this is evidence that cytochrome P-450-I is present in alveolar type II cells. The localization of cytochromes P-450 in isolated type II and Clara cells is being investigated further in our laboratory.

Thirty per cent of the cells constituting the cell digests prepared in this study were represented by alveolar type II cells. This may have been an artificial enrichment of type II cells (in cell digest versus whole lung), however, since the various proteolytic enzymes seemed to release different proportions of lung cell types. It has not yet been demonstrated what percentage of the cells in intact lung can be accounted for by alveolar type II cells. Therefore, it is impossible at this time to know what proportion of total pulmonary MFO activity is attributable to type II cells. The ratio of 7-Ec deethylase to coumarin hydroxylase in total lung homogenate and microsomes, however, is considerably higher than in the enriched Clara cell fraction. Assuming that coumarin hydroxylase is present only in the Clara cells, this suggests that other cell types besides the Clara cell are contributing a significant part of the 7-Ec deethylase activity as measured in whole lung homogenate, and the type II cells account for at least part of that activity. Although the xenobiotic metabolizing specific activities in type II cells do not seem to be enriched (on a per mg protein basis) from the cell digest, these cells could still account for a large part of the total activities seen in the whole lung.

It seemed that NADPH might be limiting for MFO activities in isolated type II cells at certain times. There was not much difference (Table 1) between the activities of unsonicated cells plus NADPH and sonicated cells plus NADPH. This difference, however, was variable (5–20 per cent between experiments). Glucose, which is necessary for the intracellular production of NADPH and is sometimes depleted during cell isolation, was present at a concentration of 5 mM in all isolation buffers. Reduction in enzyme activity related to limiting amounts of intracellular NADPH did not seem to occur in the 30% enriched Clara cell fraction, where the 7-Ec deethylase activity was consistently about the same in whole cells with or without NADPH, and in sonicated cells in the presence of NADPH.

When NADH and NADPH were compared as cofactors for 7-Ec deethylase activity, NADH proved to be an inhibitor of the reaction in sonicated alveolar type II cells. This activity in rabbit hepatic microsomes was enhanced by NADH. Sasame *et al.* [25] found that NADH stimulated the metabolism of 4-ipomeanol (IPO) in rat pulmonary microsomes when NADPH was present in the incubation and gave about 30 per cent of the activity in the absence of NADPH. Boyd [26] has localized IPO metabolism within the rat lung to the Clara cell. This suggests another possible difference between the xenobiotic metabolisms of the type II and Clara cells, although this needs further investigation.

Whenever xenobiotic metabolism was assayed in lung cells, activities in either the cell digest or lung homogenate were measured for comparison with the cell fractions. It was felt that activities in the cell digest should have been similar to or higher than (assuming enrichment of cells with high MFO activity) those in the lung homogenate. However, that was not always found to be the case. As seen in Table 1, 7-Ec deethylase was much higher in the lung homogenate than in the cell digest. One explanation seems to be protease inhibition of the cell digest and perhaps other cell fractions as well. A large part of the cell digest is composed of cell debris which could be exposed during cell preparation to the potent inhibitory action of even small amounts of protease even though the protease does not seem to affect the whole cells.

It is possible that the alveolar type II cells which have been isolated following elutriator and density gradient separations have activities lower than are found in these cells *in vivo*. It is not known whether the cell isolation procedures (other than protease treatment) selectively destroy activities in one cell type more than in another or one activity more than another.

The alveolar type II cells isolated from rabbit lung have been shown in this report to possess the components necessary for xenobiotic biotransformations as seen in pulmonary microsomal systems. Although the levels of these enzymes in type II cells appear to be lower than in Clara cells, it cannot be assumed that the cytochrome P-450-dependent MFO enzymes are located only in the Clara cell. The nature of this system in type II cells will be compared further with

the xenobiotic metabolism of isolated Clara cells in future reports.

Acknowledgements—The authors appreciate the excellent technical assistance of Ms. Anna Reddin. This research was supported, in part, by the U.S. Environmental Protection Agency under an interagency agreement relating to the Federal Interagency Energy/Environmental Research and Development Program.

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