Effects of Coal Tar Dyes on Viability, and RNA and Protein Syntheses in Isolated Rat Hepatocytes*

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The effects of Amaranth, Ponceau 3R, Sunset Yellow FCF, and Rose Bengal on viability and RNA and protein syntheses were studied in suspensions of viable rat hepatocytes. Liver parenchymal cells were enzymatically prepared from normal adult rats by perfusion of the liver with buffer solution of 0.05% collagenase, followed by shaking of liver slices in the same solution. The cell yield was $60.5 \pm 15.8 \times 10^6$ cells/g liver and the cell viability index was $87.0 \pm 2.4\%$. When the isolated hepatocytes were incubated in the presence of coal tar dyes at a concentration of 1 mM for 4 hr, a decrease in the cell viability was observed. In particular, the Rose Bengal-treated fraction showed a rapid decrease of the cell viability in comparison with other dye-treated fractions. ^3H -Uridine incorporation into RNA was stimulated in the presence of Amaranth or Ponceau 3R. On the other hand, none of the coal tar dyes tested in this study had any effect on ^{14}C -isoleucine incorporation into protein. These results suggest that the system of isolated hepatocytes may be a good model for studying the mechanism of action of toxic chemicals.

Key words: rat; isolated hepatocyte; collagenase; viability index; RNA synthesis; protein synthesis; Amaranth; Ponceau 3R; Rose Bengal; Sunset Yellow FCF

Introduction

It is now recognized that the majority of chemicals require metabolic activation for their toxic potential to be realized and the enzyme activity causing this activation is localized predominantly in mammalian hepatocytes¹⁾. Conversely, a number of chemicals are inactivated by metabolism and this activity is also preferentially localized in mammalian hepatocytes¹⁾.

Some workers have used primary hepatocyte cultures to study the metabolism of chemicals^{2)–5)}. These studies have demonstrated that the metabolism of chemicals by isolated viable hepatocytes in suspension close-

ly resembles the *in vivo* situation. Consequently, the isolated hepatocyte system appears to be a useful model for studies of the *in vivo* metabolism of chemicals. Therefore, we decided to utilize this cell culture system in studies of the metabolism of food additives.

In our previous paper⁶⁾, Ponceau 3R, Ponceau R and Amaranth have been shown to stimulate and Rose Bengal to inhibit RNA synthesis in isolated rat liver nuclei. Ponceau R and Ponceau 3R have been banned as food additives, because they have been associated with toxic effects, such as induction of liver injury and tumor formation^{7)–10)}. Since Amaranth was first suggested to have carcinogenic properties¹¹⁾, various authors have investigated the teratogenic carcinogenic, and toxic effects of Amaranth^{12)–14)}. Further, our re-

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cent study has indicated that Ponceau 3R and Amaranth stimulate RNA synthesis in rat liver after oral administration of the azo dyes¹⁵⁾.

This report describes the effects of Ponceau 3R, Amaranth, Sunset Yellow FCF, and Rose Bengal on viability and RNA and protein syntheses in isolated rat hepatocytes. Sunset Yellow FCF was previously found to have no effect on RNA synthesis in isolated rat liver nuclei⁹⁾.

Materials and Methods

Chemicals and isotopes

Collagenase (EC 3.4.4.19) was obtained from P-L Biochemicals, Inc., and fetal bovine serum from Sanko Pure Chemicals. Trypan blue was purchased from Chroma Gesellschaft. Ponceau R, Amaranth, Sunset Yellow FCF, and Rose Bengal were obtained from the National Institute of Hygienic Sciences in Japan. ¹⁴C-Isoleucine (321 mCi/mmol) and ³H-uridine (26.6 Ci/mmol) were products of New England Nuclear.

Preparation of rat hepatocyte suspension

Male rats of the Wistar strain weighing 200 g to 250 g were fed a commercial diet obtained from Wako Pure Chemicals Industries, Ltd. and water *ad libitum*. The procedure for preparing a single cell suspension was a modification of that of Yamasaki and Ichihara¹⁶⁾. The abdomen and chest of the rat were opened under ether anesthesia, and the liver was perfused via the portal vein with 30 ml of calcium- and magnesium-free Hanks' solution containing 0.5 mM ethyleneglycol-bis-(β -aminoethylether) N, N' - tetracetic acid and 20 ml of enzyme solution. The enzyme solution was 0.05% collagenase dissolved in Hanks' solution (pH 7.2) containing 2% bovine serum albumin and 10 mM HEPES. The liver lobes were cut into slices of about 1 mm in thickness with a Stadie-Riggs tissue slicer. Slices (3 g) were then placed in a 250-ml conical flask containing 10 ml of enzyme solution. All procedures to this point were carried out at 4°C. The flasks were then shaken in a shaking water bath at 37°C for 60 min (120 oscillations/min). The resulting

cloudy supernatant was mixed with 20 ml of calcium- and magnesium-free Hanks' solution and filtered through a layer of nylon cloth (60 μ m pore size; Noguchi Bolting Cloth, Tokyo) so as to remove large cell clumps and undigested tissues. The filtrate was resuspended in 10 ml of calcium- and magnesium-free Hanks' solution containing 2% bovine serum albumin, then centrifuged, and resuspended in incubation medium to give the final cell suspension.

Cell yield and viability

The viability was based on the ability of cells to exclude trypan blue. Cell suspension, 0.4 ml, was mixed with 0.2 ml of 0.5% trypan blue solution (dissolved in isotonic salt solution) and incubated at 37°C for 5 min. Stained and unstained cells were counted with a Thoma hemacytometer.

Incubation and metabolic experiment¹⁷⁾

Syntheses of RNA and protein were measured by using ³H-uridine and ¹⁴C-isoleucine as precursors, respectively. The isolated liver cells were suspended in 7 ml of Hanks' solution containing 10% fetal bovine serum (pH 7.2). The protein content of the hepatocytes was measured by the method of Lowry¹⁸⁾ with crystalline bovine albumin as a standard. The cells were shaken in an incubator at 37°C for 5 min, and the experiment was initiated by addition of dyes and labelled precursors. After the desired length of time, the incubation was terminated by pipetting an aliquot of the cell suspension into 10 ml of ice-cold isotonic saline. The cells were sedimented by centrifugation at 1,000 $\times$ g for 10 min and washed twice with 5% trichloroacetic acid solution (TCA), followed by two 15-min extractions with 5% TCA at 90°C. Tritium recovered in the supernatant fluid from the hot TCA extractions was used as a measure of RNA synthesis. The resulting precipitates were washed once with ethanol: ether (1:1) and once with ether, then digested with 0.2 N ammonium hydroxide. The ¹⁴C content of the digest was determined and used as a measure of protein synthesis. The radioactivity was counted in a Beckman LS-250 liquid scintillation counter.

Table 1. Comparison of Viable Cell Yield and Viability Index of Hepatocyte Suspensions Prepared by our Method and That of Fry *et al.*¹⁰⁾

Method used	Viable cell yield ($\times 10^6$ /g liver)	Viability index (%)
This paper	$60.4 \pm 15.8^*$ (41–80)	$87.0 \pm 2.4^*$ (84–90)
Fry <i>et al.</i> ¹⁰⁾	12.2 ± 0.7 (7.0–19.9)	84 ± 2 (70–98)

* Values are expressed as means $\pm$ S.D. of four experiments with the range in brackets

Results and Discussion

A suspension of rat hepatocytes was prepared by perfusing the liver with dissociation medium and shaking the sliced tissue in dissociation medium. The cell yield and viability of isolated hepatocytes are shown in Table 1. The cell yield of isolated hepatocytes produced by the method described in this paper was $60.4 \pm 15.8 \times 10^6$ cells/g liver. The majority ($87.0 \pm 2.4\%$) of hepatocytes was viable. These results compare very favorably with the result reported by Fry *et al.*¹⁰⁾ The viability indexes were approximately same but the method employed in this paper gave a markedly increased yield of viable rat hepatocytes. The method of Yamasaki and Ichihara¹⁰⁾ is a compromise between the shaking method of Howard *et al.*²⁰⁾ and the perfusion method of Berry²¹⁾. The only enzyme used for dissociation of liver tissue is collagenase in this method. According to Fry *et al.*¹⁰⁾, the absence of hyaluronidase (EC 3.2.1.35) appeared to decrease the viable cell yield with no loss of viability. On the other hand, Seglen reported that hyaluronidase, an enzyme commonly used in liver perfusion procedures, had no detectable dispersion activity alone and inhibited dispersion at high enzyme concentrations²²⁾. Our results also suggest that hyaluronidase is not necessary for dissociation of liver tissue. Important factors in the isolation of viable hepatocytes by the method employed in this work are the accuracy of slicing (as judged by the thickness of the slices) and the perfusion procedures. The effects of coal tar dyes on viability, and RNA and protein syntheses were studied in these cell suspensions.

The effects of duration of incubation on the viability of hepatocytes in the presence or

Fig. 1. Effects of coal tar dyes on viability of isolated rat hepatocytes. A hepatocyte suspension (7 ml) containing 2×10^7 cells/ml was incubated at 37°C for 4 hr. Each of the coal tar dyes was added to a final concentration of 1 mM. Aliquots were taken at the indicated times for the determination of viability and processed as described under "Materials and Methods". Each point is a mean of three experiments. ○, no addition of dye; ●, Sunset Yellow FCF; △, Amaranth; ▲, Ponceau 3R; □, Rose Bengal

absence of coal tar dyes is shown in Fig. 1. Each of the coal tar dyes was added to the incubation medium at a final concentration of 1 mM. When the hepatocytes were incubated in the absence of coal tar dyes, a decrease of the cell viability was observed and the viability index fell to 87% to 75% after 4 hr. The viability index of Sunset Yellow FCF- or Amaranth-treated hepatocytes was approximately 65% after 4 hr. When the hepatocytes were incubated in the presence of Ponceau 3R, the viability index fell to about 50% after 4 hr. A rapid decrease of viability of Rose Bengal-treated hepatocytes was observed, and after 4 hr no viable cells could be found.

Fig. 2. Effect of coal tar dyes on ^3H -uridine incorporation into RNA in isolated rat hepatocytes

A hepatocytes suspension (7 ml) containing 2×10^7 cells/ml was incubated with $5 \mu\text{Ci}$ ^3H -uridine at 37°C for 2 hr. Each of the coal tar dyes was added to a final concentration of 1 mM. Aliquots of 0.5 ml were taken at the indicated times and processed as described under "Materials and Methods". Each point is a mean of three experiments.

▲, Ponceau 3R; △, Amaranth; ○, no addition of dye; ●, Sunset Yellow FCF; □, Rose Bengal

Incorporation of ^3H -uridine into RNA by the hepatocytes in the presence or absence of coal tar dyes is shown as a function of time in Fig. 2. The incorporation was measured during incubation for up to 2 hr. No difference in the incorporation of ^3H -uridine into RNA could be detected between the control and Sunset Yellow FCF- or Rose Bengal-treated hepatocytes. Both Amaranth- and Ponceau 3R-treated hepatocytes showed higher incorporation than the control.

Incorporation of ^{14}C -isoleucine into protein of hepatocytes in the presence or absence of coal tar dyes is shown in Fig. 3. None of the coal tar dyes tested had any effect on the incorporation of ^{14}C -isoleucine into protein.

We have reported that Rose Bengal inhibited RNA synthesis in isolated liver nuclei⁹. In isolated rat hepatocytes, however, this dye had no effect on RNA or protein synthesis. These results suggest that Rose Bengal may not be able to affect the components of nuclei through the barrier of the cytoplasm at the

Fig. 3. Effect of coal tar dyes on ^{14}C -isoleucine incorporation into protein in isolated rat hepatocytes

A hepatocyte suspension (7 ml) containing 2×10^7 cells/ml was incubated with $3 \mu\text{Ci}$ ^{14}C -isoleucine at 37°C for 2 hr. Each of the coal tar dyes was added to a final concentration of 1 mM. Aliquots of 0.5 ml were taken at the indicated times and processed as described under "Materials and Methods". Each point is a mean of three experiments.

○, no addition of dye; ●, Sunset Yellow FCF; □, Rose Bengal; ▲, Ponceau 3R; △, Amaranth

dye concentration used (1 mM). Several authors have reported that Rose Bengal strongly binds to various kinds of enzymes and inhibits their activities^{9, 23, 24}. Work is in progress to investigate whether Rose Bengal inhibits RNA synthesis in the liver after oral administration of this dye to rats.

Both Amaranth and Ponceau 3R stimulated RNA synthesis in isolated hepatocytes (Fig. 2). We previously found that both azo dyes stimulate RNA synthesis in rat liver after oral administration¹⁵. The results obtained from studies in isolated hepatocytes thus reflect the *in vivo* ones. On the other hand, the incorporation of radioactive orotic acid or adenine *in vitro* and *in vivo* is increased in the precancerous liver by prolonged feeding of a carcinogenic azo dye, N,N-dimethyl-4-aminoazobenzene²⁵⁻²⁷. Further, Wu and Smuckler have reported that the administration of the carcinogenic azo dye results in enhanced RNA formation, enhanced chromatin binding of the RNA polymerase and

the expression of previously repressed genomic segments²⁸⁾, indicating that the azo dye may alter genetic expression by epigenetic means. However, the processes by which these changes are brought about are not known. Our previous paper²⁰⁾ has shown that the chromatin of isolated rat liver nuclei treated with Amaranth and Ponceau 3R is highly susceptible to digestion by DNase I, suggesting that addition of the azo dyes may change the transcriptionally inactive form into the transcriptionally active form in nuclear chromatin. Many investigators have reported that the transcriptionally active chromatin is extremely susceptible to digestion by DNase I³⁰⁾. Moreover, our recent study has demonstrated that both Ponceau 3R and Amaranth stimulate RNA synthesis by causing the dissociation of heterochromatin in isolated rat liver nuclei^{*1}.

Recent studies of chemical carcinogenesis have demonstrated that potential transforming genes are encoded in the genomes of normal mammalian cells^{31,32)}. These data suggest that Ponceau 3R and Amaranth may activate the transforming genes that are repressed in the genomes of normal cells.

In conclusion, we investigated the use of isolated hepatocytes for studying the effects of coal tar dyes on viability, and RNA and protein syntheses. There are some limitations in this system. However, our results suggest that this system may be a good model of the liver for studying the action of food additives.

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