EFFECT OF MICROCYSTIN-LR ON CULTURED RAT ENDOTHELIAL CELLS

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Primary cultures of adult rat hepatic sinusoidal endothelial cells were used to investigate the effect of microcystin-LR. Microcystin-LR at a concentration (4 μM), which induces necrosis in cultured rat hepatocytes, did not produce either permeability changes, or cytotoxicity in endothelial cell monolayers. However, supernatants derived from cultured rat hepatocytes treated with 4 μM microcystin-LR induced significant permeability changes, as indicated by the release of [^{14}C]adenine nucleotides, and a small reduction of cell density in endothelial cell monolayers. Silymarin at 0.2 mM but not dithioerythritol at 2.5 mM, partially protected changes in endothelial cells produced by supernatants derived from microcystin-LR-treated hepatocytes. Thus, the effect of microcystin-LR on liver sinusoidal endothelial cells *in vitro* was an indirect one; hepatocytes treated with microcystin-LR produced either an activated metabolite(s) or other factors that affected endothelial cells. Indirect endothelial cell injury may contribute to microcystin-LR-induced liver hemorrhage observed *in vivo*. (JES)

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R. SOLOW, K. MEREISH, G.W. ANDERSON, JR. and J. HEWETSON. Effect of Microcystin-LR on Cultured Rat Endothelial Cells. *Toxicon* , , 1989. Primary cultures of adult rat hepatic sinusoidal endothelial cells were used to investigate the effect of microcystin-LR. Microcystin-LR at a concentration (4 μM), which induces necrosis in cultured rat hepatocytes, did not produce either permeability changes, or cytotoxicity in endothelial cell monolayers. However, supernatants derived from cultured rat hepatocytes treated with 4 μM microcystin-LR induced significant permeability changes, as indicated by the release of [^{14}C]adenine nucleotides, and a small reduction of cell density in endothelial cell monolayers. Silymarin at 0.2 mM but not dithioerythritol at 2.5 mM, partially protected changes in endothelial cells produced by supernatants derived from microcystin-LR-treated hepatocytes. Thus, the effect of microcystin-LR on liver sinusoidal endothelial cells *in vitro* was an indirect one; hepatocytes treated with microcystin-LR produced either an activated metabolite(s) or other factors that affected endothelial cells. Indirect endothelial cell injury may contribute to microcystin-LR-induced liver hemorrhage observed *in vivo*.

INTRODUCTION

Microcystin-LR, a seven-amino acid cyclic polypeptide synthesized by the cyanobacterium *Microcystis aerugenosa*, induces hepatotoxicity in many species including man (GORHAM and CARMICHAEL, 1979; JACKSON *et al.*, 1985). After administration to laboratory rodents, microcystin-LR rapidly induces severe liver hemorrhage, which is associated with centrilobual hepatocyte necrosis (SCHWIMMER and SCHWIMMER, 1964; THEISS and CARMICHAEL, 1986). Microsystin-LR not only induces the rapid onset of liver damage in rodents *in vivo*, but also induces necrosis of cultured rat hepatocytes after several hours of incubation with the toxin (FOXALL and SASNER, 1981). The mechanism by which microcystin-LR induces hepatotoxicity is not known. Microcystin-LR effects do not appear to be mediated by the inhibition of macromolecular biosynthesis; i.e. protein, RNA or DNA synthesis (RUNNEGAR and FALCONER, 1981). Microcystin-LR does however, induce early changes in cultured hepatocytes, such as cell deformation (blebbing), rapid rise in intracellular calcium, increased phosphorylase-a activity, depletion of glutathione (RUNNEGAR *et al.*, 1987; FALCONER and RUNNEGAR, 1987) and the release of arachidonic acid metabolites (NASEEM *et al.*, 1988). These early events were followed by the leakage of adenine nucleotides and, cytoxolic enzymes and eventually, the loss of cell viability (MEREISH *et al.*, 1989).

Although microcystin-LR toxicity to cultured hepatocytes has been well documented, relatively little is known about its effects on other non-parenchymal liver cells, that is, sinusoidal endothelial and Kupffer cells. In the present study, we investigated the effects of microcystin-LR on cultured primary liver endothelial cells. We also investigated whether hepatocytes treated with a cytotoxic dose (4 μM) of microcystin-LR release factors (mediators) that could induce changes, or cytotoxicity in endothelial monolayers. Some endothelial cells were pre-treated with the anti-oxidants, dithioerythritol (DTE) (CLELAND, 1964), or silymarin (SM) (FRAGA *et al.*, 1987), in order to determine if these agents could prevent changes induced by supernatants derived from microcystin-LR treated hepatocytes. [^{14}C]Adenine nucleotide release was used to monitor cell damage (SHIRHATTI and KRISHNA, 1985). While the release of [^{14}C]adenine nucleotides from healthy endothelial cells is relatively high, it is a sensitive method for detecting small perturbations or metabolic disturbances in endothelial cells (PEARSON and GORDON, 1979). Microscopy and cell density assays were also used to detect changes in the endothelial cells.

MATERIAL AND METHODS

Materials

The following materials were obtained commercially from the indicated sources: silymarin (SM) (Aldrich Chemical Co. Inc., Milwaukee, WI), [^{14}C]adenine (60 mCi/mmol, New England Nuclear Corp., Boston, MA), tissue culture media and fetal bovine serum albumin (Gibco, Grand Island, NY), tissue culture ware (Becton-Dickinson Labware, Lincoln Park, NJ), collagen, collagenase type IV, dithioerythritol (DTE), endothelial growth factor and heparin (Sigma Chem. Co., St. Louis, MO).

Male, WF.LEW inbred rats (G. Anderson, USAMRIID, Fort Detrick, Frederick, MD), weighing 250-300 g, were used for all experiments. Microcystin-LR, assessed as > 85% pure by high-performance-liquid-chromatography was obtained from Dr. W. Carmichael, Wright State University, Dayton, OH.

Hepatocytes

Rat hepatocytes were isolated and cultured according to the method of ELLIGET and KOLAJA (1983). Hepatocytes were separated from non-parenchymal cells by low-speed centrifugation (500 X g) for 35-45 sec. After washing the hepatocyte pellet several times, the number of viable cells was determined by trypan blue exclusion. Hepatocytes were suspended at 5 x 10^5 viable cells per ml in Leibovit's (L15) culture medium containing 17% fetal bovine serum (FBS) and seeded in collagen-coated, 35 mm, 6-well-plates by adding 1 ml of the cell suspension per well. The cells were allowed to attach for 30 min at room temperature and then incubated at 37°C with 5% CO_2 and 90% humidity for an additional 2 hr. After incubation, the majority of hepatocytes attached and established a monolayer. Non-attached cells were removed by aspiration and an additional 1 ml of culture medium was added to each well.

Sinusoidal endothelial cells

Endothelial cells were isolated according to the procedure of SMEDSROD and PERTOFT (1985). Non-parenchymal cells isolated from *in situ* liver perfusion were layered over a two-step Percoll gradient and centrifuged for 15 min at 4°C at 800 X g. After centrifugation, sinusoidal endothelial cells were collected at the interface between the two Percoll gradients. The endothelial cells were washed twice at 700 x g for 5 min in RPMI 1640 medium. The number of viable endothelial cells was determined by trypan blue exclusion. Endothelial cells were resuspended at 0.5 x 10^6 viable cells per ml in RPMI 1640, containing 10% FBS and 15 mg of endothelial growth factor, and seeded on collagen-coated, 35 mm, 6-well-plates by adding 1 ml of cell suspension per well. The cells were incubated overnight at 37°C with 5% CO_2 and 90% humidity. After incubation, the majority of endothelial cells had attached and established a monolayer. Non-atttached endothelial cells were removed by aspiration and an addition 1 ml of culture medium was added to each well.

Treatment of hepatocytes with microcystin-LR

One ml of L15 medium containing 4 μM of microcystin-LR or 1 ml of medium alone was added to hepatocyte monolayers 3 hr post plating. The cells were incubated for 16 hr (overnight) at 37°C in a humdified incubator in the presence of 5% CO_2. After incubation, cell supernatants were removed from both the treated and control cells and centrifuged at 500 X g for 4 min in an Eppendorf centrifuge, Model-5414, to remove cell debri. Supernatants from both treated and non-treated hepatocytes were pooled separately and stored at 4°C for a maximum of 2 hr before use.

Labeling and treatment of endothelial cells

After endothelial cells were incubated overnight, the culture medium from each well was replaced with 1 ml of RPMI 1640 containing [^{14}C]adenine (1.4 μM, 0.082 μCi). The cells were incubated for 2 hr at 37°C in a humidified incubator in the presence of 5% CO_2. After incubation, the [^{14}C]adenine-containing medium was removed and the cells were washed with RPMI 1640. These labeled cells were then incubated with 1 ml of L15 medium containing 4 μM microcystin-LR, medium alone, or 1 ml of supernatants from control or microcystin-LR-treated hepatocytes for a total of 20 hr. In addition, some endothelial cells were pre-treated for 30 min with 2.5 mM DTE or 0.2 mM SM followed by the addition of supernatants derived from microcystin-LR-treated hepatocytes or L15 medium.

In order to determine the amount of [^{14}C]adenine nucleotides released from control and treated cells during incubation, 0.1 ml of cell supernatants was removed from each well at selected time intervals and counted in 10 ml of Hydrofluor (National Diagnostic, Summerville, NJ) in a Beckman Scintilation Counter, Model LS5800 (Beckman Inst. Co., Fullerton, CA). After 20 hr of incubation, the cells were lysed by the addition of 1 ml of 0.05% digitonin in phosphate buffer to each well. An aliquot of each cell lysate was used to measure cellular [^{14}C]adenine nucleotides and protein content. Protein levels were determined by using Pierce protein reagent (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Cell morphology during the incubation period was assessed by light microscopy with a Nikon Diphot inverted phase contrast microscope. Photographs were taken with a Nikon FE camera with Tungsten 50, 35-mm, color slide film. Photographs of cells were subjected to quantitative analysis to describe differences in cell morphology. Changes in cell morphology were manually measured in mm from 25 X 20 cm photographs for total length (L) (cell body and projection(s)), cell body length (L_B) and cell width (w). The photograph was divided into 5 equal areas, where 10 cells were randomly selected from each area. The selected fifty cells from each photograph were measured. Data were analyzed for statistical difference using t-test (a=0.05) between two population means (Ott, 1981).

RESULTS

The release of [^{14}C]adenine nucleotides from endothelial cells treated with 4 μM microcystin-LR was not significantly different from control cells incubated with L15 medium alone (Fig. 1). Concentrations of up to 50 μM microcystin-LR did not induce significant release of nucleotides over control levels (data not shown).

Endothelial cells treated with supernatants from untreated hepatocytes (Fig. 2) released less [^{14}C]adenine nucleotides than control cells incubated in L15 medium alone (Fig. 1). These healthy, control hepatocytes probably released into the medium "conditioning factors" which were beneficial to endothelial cells. Endothelial cells, however, incubated with supernatants derived from hepatocytes treated with 4 μM microcystin-LR, released significantly more [^{14}C]adenine nucleotides than cells incubated with control supernatants (Fig. 2). The difference in [^{14}C]nucleotide release between the treated and untreated cells was observed as early as 3 hr post-exposure and continued for 20 hr. Endothelial cells treated with 2.5 mM DTE and then incubated with supernatants derived from microcystin-LR-treated hepatocytes released the same amount of [^{14}C]adenine nucleotides as did cells treated with microcystin-LR supernatants. However, cells pre-treated with SM, then with microcystin-LR supernatants, released statistically significantly lower levels of [^{14}C]adenine nucleotides than were induced by microcystin-LR supernatants alone (Fig. 2).

The morphology of endothelial cells after 4 hr exposure to microcystin-LR, or hepatocyte supernatants is shown in Figure 3. Endothelial cells incubated with L15 medium alone, or microcystin-LR alone (Fig. 3a), or supernatants derived from untreated hepatocytes displayed similarly a spindle-shape cell with extended, cytoplasmic processes to neighboring cells ($L = 10.5 \pm 5.5$; $L_B = 3.5 \pm 1.1$; $W = 1.8 \pm 0.5$, in mm). Endothelial cells, however, incubated with supernatants derived from microcystin-LR-treated hepatocytes

(Fig. 3b), displayed rounded, more contracted cell bodies with fewer extensions ($L = 9.9 \pm 6.3$; $L_B = 2.9 \pm 1.0$; $W = 2.0 \pm 0.7$, in mm). Statistical difference (t-test, a=0.05) were only observed in L_B.

Endothelial cell densities were determined by protein levels (in culture wells) measured 20 hr after incubation. There was no statistical significant difference (t-test, a=0.05) in protein content (mg/ml) between endothelial cell monolayers treated with L15 medium alone (0.213 ± 0.041), 4 µM microcystin-LR (0.176 ± 0.052) or supernatants derived from control (0.144 ± 0.019) and treated (0.119 ± 0.008) hepatocytes.

DISCUSSION

The release of [^{14}C]adenine nucleotides from control endothelial cells incubated in either L15 medium or with supernatants from untreated hepatocytes was similar to that released from porcine aortic endothelial cells labeled with [^{3}H]adenosine (LEROY *et al.*, 1984; PEARSON *et al.*, 1978). Endothelial cell monolayers incubated directly with the toxin exhibited the same morphological features, and cell density, and released the same amounts of [^{14}C]adenine nucleotides as control cells treated with L15 medium alone. These observations indicate that microcystin-LR did not directly injure primary sinusoidal liver endothelial cells. However, supernatants derived from microcystin-LR-treated hepatocytes did induce significant changes in cultured endothelial cell monolayers. These changes include significant increase in [^{14}C]adenine nucleotide release, rounded or contracted cell shape, and a small reduction in cell density. The release of [^{14}C]nucleotides combined with the changes in cell shape (which occurred during approximately the same time) suggests that supernatants derived from microcystin-LR-treated hepatocytes induced an increase in the permeability of endothelial cells.

Permeabilty changes in endothelial cells are thought to be due to changes in cell shape mediated by a calcium-dependent contraction of actin-myosin microfilaments combined with the activation of actin-severing proteins, i.e., gelsolin, fragmin and villin (SHASBY *et al.*, 1982; SAVION *et al.*, 1982; WEEDS, 1982). The contraction of cytoskeletal microfilaments induces the loss of cell-to-cell junctions and creates gaps between adjacent cells (MAJINO *et al.*, 1967; FANTONE *et al.*, 1980).

It is possible that supernatants derived from microcystin-LR-treated hepatocytes contain either active microcystin-LR metabolite(s) or cell products that affect endothelial cells. In fact, microcystin-LR has been shown to induce the release of arachidonic acid metabolites in cultured hepatocytes (NASEEM *et al.*, 1988). Arachidonic acid is known to stimulate prostaglandin synthesis in cultured endothelial cells (BORDET *et al.*, 1986). Microcystin-LR-treated hepatocytes have also been shown to release adenine nucleotides (ATP, ADP, AMP) and adenosine (MEREISH *et al.*, 1989), which act as local hormones in increasing the permeabilty of the microvasculature (PEARSON and GORDON, 1979). The release of adenine nucleotides from damaged hepatocytes could therefore play a role in the initiation of liver hemorrhage followed by permeabilty changes in endothelial cells, thereby creating red cell extravasation.

SM lessened the endothelial cell changes induced by supernatants from microcystin-LR-treated hepatocytes. Both DTE and SM have been shown to protect cultured hepatocytes against a variety of hepatotoxic agents (NICOTERA *et al.*, 1985; WAGNER, 1986; HIKINO *et al.*, 1984) including microcystin-LR (MEREISH and SOLOW, 1989). SM provides protection possibly through its known combined action of scavaging free radicals (VALENZUELA *et al.*, 1986), inhibiting lipoxygenation and, therefore, leukotriene synthesis (BAUMANN *et al.*, 1980).

It could be argued that the permeablity changes produced in endothelial cells by supernatants from microcystin-LR-treated hepatocytes are just the response of endothelial cells to the release of contents of dead hepatocytes. Experiments are now in progress to test this possibility. It is more likely, however, that the factors responsible for producing changes in endothelial cells are induced as a consequence of the interaction of microcystin-LR with hepatocytes. The increase of endothelial cell permeability induced by factors released by microcystin-LR-treated hepatocytes may contribute secondarily to the toxicity of microcystin-LR *in vivo*.

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FIG. 1. EFFECT OF MICROCYSTIN-LR ON THE PERCENT RELEASE OF [^{14}C]ADENINE NUCLEOTIDES FROM PRIMARY SINUSOIDAL ENDOTHELIAL MONOLAYERS. Endothelial cells (5×10^5 /ml) were incubated with [^{14}C]adenine (1.4 μM, 0.081 μCi) for 1 hr at 37°C. The cells were then washed and reincubated in 1 ml L15 medium containing 4 μM microcystin-LR (□--□) or medium alone (o—o), as described in the text, for 20 hr. At selected time intervals, supernatants were collected and the amount of [^{14}C]nucleotides released from endothelial cells were determined (mean ±SD, n=3). There was no statistical difference (AOV, α = 0.05 for F-test) between treatments.

FIG. 2. EFFECT OF SUPERNATANTS DERIVED FROM MICROCYSTIN-LR-TREATED HEPATOCYTES ON THE PERCENT RELEASE OF [^{14}C]ADENINE NUCLEOTIDE FROM PRIMARY SINUSOIDAL ENDOTHELIAL MONOLAYERS. [^{14}C]Adenine endothelial cells were incubated with supernatants collected from hepatocytes incubated with 4 μM microcystin-LR (□--□) or medium alone (o—o). Some endothelial cells were treated with either 2.5 mM DTE (■--■) or 0.2 mM SM (●--●) 30 min before receiving supernatants from microcystin-treated hepatocytes. Endothelial cells were incubated for a total of 20 hr and the amount of [^{14}C]adenine nucleotides released was determined (mean ± SD, n=3). SM treatment (●--●), but not DTE (■--■), was statistically significant (AOV, α = 0.05) from microcystin-LR (□--□) treatment. All treatments were statistically significant (AOV, α = 0.05) from medium control (o—o).

FIG. 3. PHASE CONTRAST MICROGRAPH REPRESENTATIVE OF RAT PRIMARY SINUSOIDAL ENDOTHELIAL CELLS 4 HR AFTER EXPOSURE TO (A) 4 μM MICROCYSTIN-LR IN L15 MEDIUM OR (B) 4 μM MICROCYSTIN-LR TREATED HEPATOCYTES. ENDOTHELIAL CELLS EXPOSED TO L15 MEDIUM ALONE OR TO SUPERNATANT DERIVED FROM CONTROL UNTREATED HEPATOCYTES WERE SIMILAR TO THAT IN (A).

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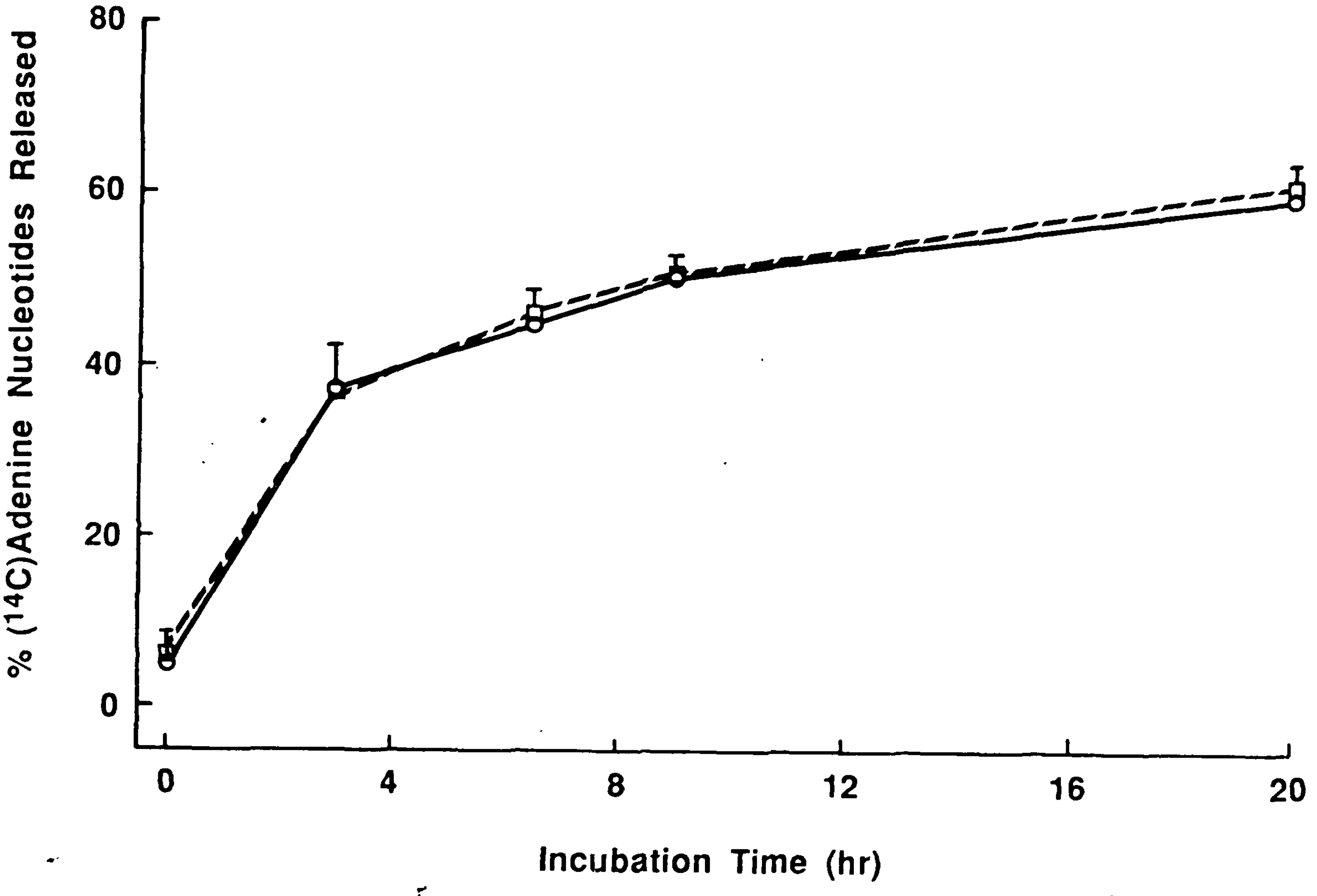

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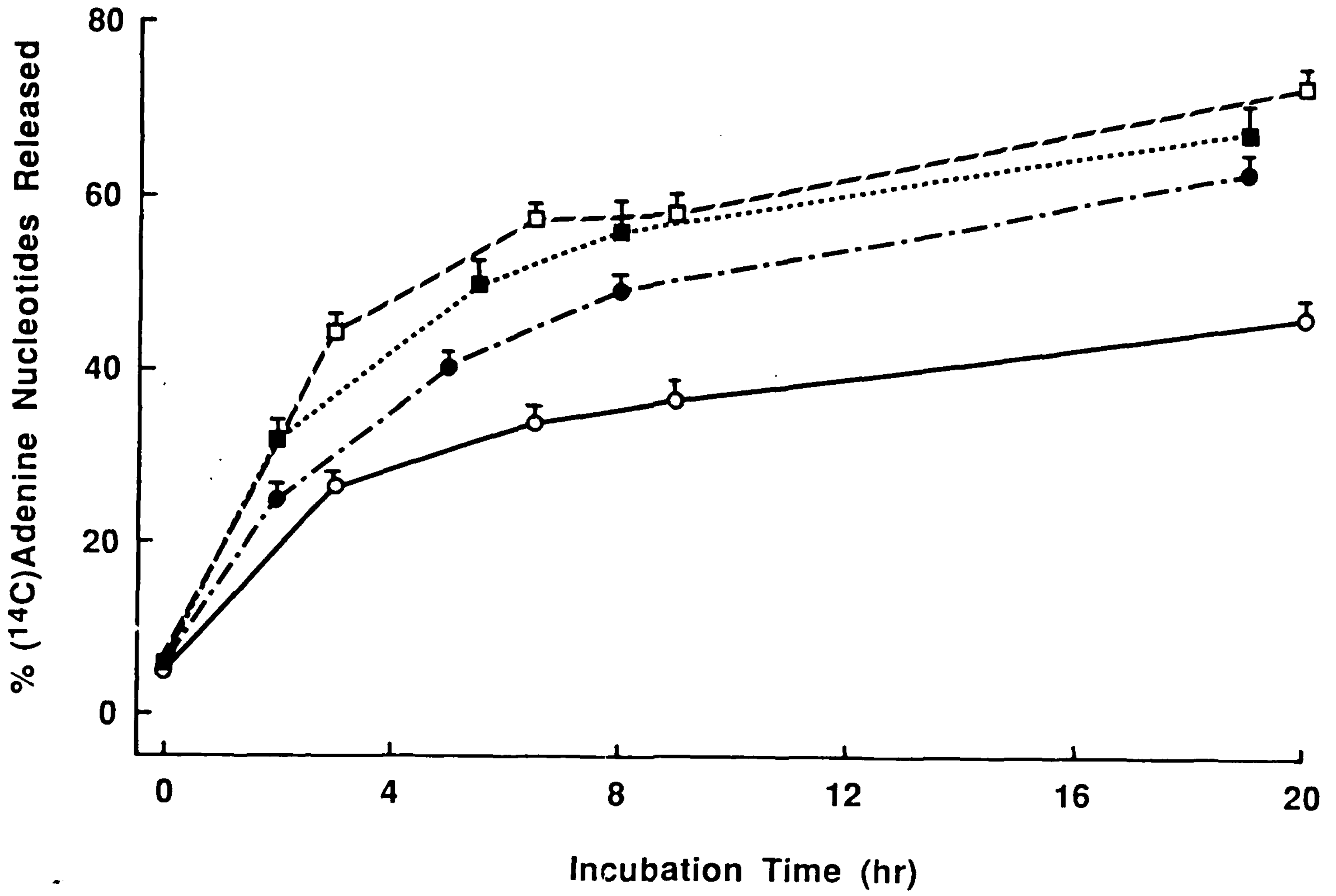
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