*Biochemical Pharmacology*, Vol. 42, No. 6, pp. 1195–1201, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00


# ALTERATIONS IN PROTEIN SYNTHESIS IN RAT LIVER CELLS BY *IN VITRO* AND *IN VIVO* EXPOSURE TO 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN

YASUNOBU AOKI,*†‡ ELLEN K. SILBERGELD,† STEPHEN R. MAX§ and BRUCE A. FOWLER†

*National Institute for Environmental Studies, Onogawa, Tsukuba, Ibaraki 305, Japan; and †Program in Toxicology and §Department of Neurology, School of Medicine, University of Maryland, Baltimore, MD 21201, U.S.A.

(*Received* 18 *February* 1991; *accepted* 26 *April* 1991)

**Abstract**—Alterations in protein synthesis in rat liver cells were examined following *in vitro* and *in vivo* exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Primary cultured rat liver parenchymal cells were exposed to 1 nM TCDD for 23 and 47 hr. Synthesis of two proteins with molecular weights ($M_r$) of 26,000 and 39,000 (designated 26k-P and 39k-P, respectively), other than cytochrome P450, was increased markedly in the cells. These proteins did not have the same antigens as cytochromes P450IA1 and P450IA2. Synthesis of three proteins with $M_r$s of 24,000, 25,000 and 29,000, respectively, was decreased by TCDD. TCDD was administered to rats at a dose of 100 μg/kg body weight. The amount of five proteins (two proteins with $M_r$ of 26,000, one of 36,000 and two of 39,000) was increased in TCDD-treated rat liver. However, the proteins increased *in vivo* by TCDD were distinguishable from 26k-P and 39k-P by two dimensional gel electrophoresis.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD‖) binds to an intracellular arylhydrocarbon receptor [1, 2] which is then translocated to the cell nucleus where it associates with specific responsive elements on the genome [3–5]. One consequence of this association is the expression of cytochrome P450 [6, 7], and the increase in UDP-glucuronosyltransferase [8], NAD(P)H:menadione oxidoreductase [9] and other xenobiotic metabolizing enzyme activities [6, 10–12]. TCDD is a very potent teratogen [13] and carcinogen [14, 15] in experimental animals, and related compounds are also suspected to be carcinogenic on the basis of epidemiological studies [16]. Gene products induced by TCDD, other than cytochrome P450 and related enzymes, are also possibly responsible for carcinogenicity, teratogenicity and other biological activities attributed to TCDD. Identification of the gene products induced by TCDD is essential for an understanding of the biological activities of TCDD. However, proteins altered in synthesis by TCDD have not been searched for systematically.

The present study examined the alteration in protein synthesis induced by *in vitro* and *in vivo* exposure to TCDD using SDS–polyacrylamide gel electrophoresis and two dimensional (2D) gel electrophoresis. 2D gel electrophoresis is a powerful tool for identifying proteins whose synthesis is altered by exposure to chemicals because several hundred proteins can be separated on one gel [17]. This study shows that the synthesis of several proteins, not documented previously, was altered in rat liver parenchymal cells following *in vitro* exposure to TCDD. After *in vivo* administration of TCDD, the amount of five proteins other than cytochrome P450 was increased in rat liver.

## MATERIALS AND METHODS

*Materials.* TCDD and anti-rat cytochrome P450IA1 rabbit immunoglobulin G (IgG) were kind gifts of Dr J. McKinney (National Institute of Environmental Health Science) and Drs Y. Takahashi and S. Miura (National Institute for Environmental Studies, Japan), respectively. Methylated $^{14}$C-labeled proteins were purchased from NEN (Boston, MA).

*Preparation of monolayer cultured rat liver parenchymal cells.* The liver of a male Fisher rat (about 150 g body wt) was perfused with collagenase solution *in situ* and liver parenchymal cells were isolated from the digested liver by the method of Seglen [18]. Viability of isolated cells was determined by trypan blue exclusion as described previously [19]; viability of cells was over 90%. The isolated cells ($0.85 \times 10^6$ cells) were plated in a plastic 6-well plate (35 mm diameter; Nunc, Kampstrup, Denmark) with 1.5 mL of Williams' E medium (Sigma Chemical Co., St Louis, MO) containing 10% (v/v) fetal calf serum (Hazleton, Denver, PA), 1 μM insulin, 10 mM

‡ To whom all correspondence should be addressed.

‖ Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; EBSS, Earle's balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin G; 2-ME, 2-mercaptoethanol; MEM, minimum essential medium; $Me_2SO$, dimethyl sulfoxide; $M_r$, molecular weight; NEPHGE, non-equilibrium pH gradient electrophoresis; SDS, sodium dodecylsulfate; TBS, 20 mM Tris–HCl buffer (pH 7.5 at 25°) containing 0.5 M NaCl; 26k-P, 39k-P and 54k-P, proteins with $M_r$ of 26,000, 39,000 and 54,000, respectively, induced in the cells by TCDD; 24k-P, 25k-P and 29k-P, proteins whose synthesis was inhibited by TCDD exposure; 2D gel, two dimensional gel.

sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin and 25 mM Hepes/NaOH buffer (pH 7.4), and cultured in a humidified incubator at 37° under 5% $CO_2$/95% air. Plastic plates were precoated with 5% (v/v) Vitrogen (Collagen Corp., Palo Alto, CA) in Dulbecco modified minimum essential medium (MEM; Gibco, Grand Island, NY) for 5 hr at 37° and then kept at 4° until use. The medium was changed 4 hr after plating to remove non-adhered cells.

*Exposure of TCDD to the cells.* After incubation for 20 hr, the cells were flattened and aggregated as reported previously [19]. The cells were exposed to TCDD by changing the medium to one containing 1 nM TCDD prepared immediately before use from 1 μM TCDD dissolved in dimethyl sulfoxide ($Me_2SO$). Control cells were exposed to 0.1% $Me_2SO$-containing medium. After exposure to TCDD for 23 or 47 hr, the cells were washed twice with Earle's balanced salt solution (EBSS, Gibco) and then incubated in MEM Eagle's medium deficient in methionine (Sigma) containing 100 μCi/mL [$^{35}$S]methionine (800 Ci/mmol, NEN), 10 mM Hepes/NaOH buffer (pH 7.4), 10% (v/v) fetal calf serum, dialysed against Dulbecco's phosphate-buffered saline, and 1 nM TCDD or 0.1% $Me_2SO$ for control, for 1 hr. After washing with EBSS three times, the cells were lysed by the addition of 0.2 mL of a lysis solution (1% Nonidet P-40 containing 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride) and the lysate was centrifuged at 2000 *g* for 5 min. The resulting supernatant (cell lysate) was stored at −70°. Radioactivity in trichloroacetic acid precipitate of the cell lysate was measured by the method of Caltabiano *et al.* [20].

In vivo *administration of TCDD to rats and preparation of crude extract from a rat liver.* TCDD was solubilized in sesame oil (Sigma) and administered perorally by gavage to six rats (male Fisher strain, 150 g body wt) at a dose of 100 μg/kg body wt in 0.1 mL/100 g body wt as described previously [21]. The same volume of sesame oil was administered to six control rats. On the 14th day after administration, the rats were killed under anesthesia by diethyl ether and their livers were perfused with saline *in situ* and stored at −70° until use. The liver was homogenized with 3 volumes of 9 M urea containing 2% (v/v) Nonidet P-40, 2% (v/v) 2-mercaptoethanol (2-ME) and 2% (w/v) Ampholine (pH 9–11; Pharmacia/LKB, Uppsala, Sweden) in a glass–Teflon homogenizer by the method of Anderson *et al.* [22]. The homogenates were centrifuged at 100,000 *g* for 30 min and the resulting supernatant (crude extract) was stored at −70° until use.

*Polyacrylamide gel electrophoresis.* Conventional SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli [23]. The cell lysate containing radioactivity of 200,000 cpm was mixed with reagents to give 125 mM Tris–HCl buffer (pH 6.8 at 25°), 2% (v/v) 2-ME, 0.1% SDS, 10% (v/v) glycerol and 0.05% bromophenol blue. Sample solution was applied to an SDS–polyacrylamide gel (16 × 14 cm, 0.75 mm in thickness) consisting of a separating gel containing 11% (w/v) acrylamide and a stocking gel with 15 sample wells (5 mm in width). 2D gel electrophoresis was performed using non-equilibrium pH gradient electrophoresis (NEPHGE) for the first dimension by the method of O'Farrell *et al.* [17]. A monomer solution [10 mL, 4% (w/v) (acrylamide/bisacrylamide (w/w) 20:1] containing 9 M urea, 2% (v/v) Nonidet P-40 and 2% (w/v) Ampholine (pH 3.5–10)] was polymerized in a glass tube (12 cm × 1.5 mm diameter) by adding 10 μL of 10% (w/v) ammonium persulfate and 7 μL of *N,N,N′,N′*-tetramethylethylenediamine. The polymerized first dimension gels were set in the vertical tube gel electrophoresis unit of which the lower chamber was filled with 20 mM NaOH and the upper chamber with 10 mM $H_3PO_4$. The cell lysate containing radioactivity of 300,000 cpm and/or the crude extract from rat liver (200 μg protein) were diluted to 10 μL with a lysis solution, mixed with 5.5 mg of urea, 0.8 μL of 2-ME and 0.8 μL of 40% (w/v) Ampholine (pH 3.5–10), and applied to the gel. Five microlitres of 4.5 M urea containing 0.5% pH 5–7 and 0.5% pH 3.5–10 Ampholine were loaded onto the sample. NEPHGE was performed for 4 hr at 400 V. After completion, the first dimension gels were taken out of the tubes and incubated in 63 mM Tris–HCl buffer (pH 6.8 at 25°) containing 2% (v/v) 2-ME, 1% SDS and 10% (v/v) glycerol at room temperature for 20 min. The gels were then rinsed with distilled water, loaded onto 11% (w/v) SDS–polyacrylamide gel (1.5 mm thick) and fixed with 1% agarose gel containing 125 mM Tris–HCl buffer (pH 6.8 at 25°) and 0.1% SDS. Second dimensional gel electrophoresis was performed at 30 mA/gel at 15°. $^{35}$S-Labeled proteins separated on the gel were detected by fluorography. After electrophoresis, the gel was fixed in a fixing solution (acetic acid/methanol/distilled water, 1:3:6), immersed in Enhance (NEN) for 2 hr and washed with distilled water. Dried gels were exposed to XOmat AR film (Kodak, Rochester, NY) for 18 hr and 4 days for conventional SDS–polyacrylamide gel and 2D gel, respectively. Proteins on the gel were stained with Coomassie Brilliant Blue R-250 by the method of Fairbanks *et al.* [24]. The mixtures of cell lysates and liver crude extracts were applied to the 2D gel to determine which proteins were identical from the two sources. The dried gel was exposed directly to X-ray film after proteins on the gel had been stained.

*Procedure of western blotting and immunochemical detection of cytochrome* P450. Western blotting and immunochemical detection were performed by the method of Towbin *et al.* [25]. After the proteins in the cell lysates were separated on an SDS–polyacrylamide gel (0.75 mm thick), they were transferred electrophoretically to a nitrocellulose membrane (Sartorius, Göttingen, Germany). Transfer of the proteins was carried out at a constant 60 V for 18 hr at 4° in a transfer chamber (Hoefer, San Francisco; width between electrodes, 6 cm) filled with an electrode buffer [methanol/25 mM Tris–190 mM glycine (pH 8.5) (1:4, v/v)]. The membrane was washed with distilled water and dried membrane was exposed directly to the X-ray film to detect $^{35}$S-labeled proteins before immunochemical staining. The membrane was blocked with 20 mM Tris–HCl buffer (pH 7.5 at 25°) containing 0.5 M NaCl (TBS)

with 1% bovine serum albumin (fraction V, Sigma) overnight and then incubated in TBS with 0.1 mg/mL anti-rat cytochrome P450IA1 rabbit IgG and 1% bovine serum albumin for 1 hr at 37°. After washing with distilled water briefly and TBS for 5 min four times, the membrane was incubated with a second antibody solution [anti-rabbit IgG goat IgG conjugating horseradish peroxidase (Bio-Rad, Richmond, CA) diluted 3000-fold in TBS with 1% bovine serum albumin] for 1 hr at 37°. Following washing with distilled water and TBS, again (as described above), the color was developed by incubation with 50 mM Tris–HCl buffer (pH 7.5 at 25°) containing 200 mg/L 3,3′-diaminobenzidine-HCl and 170 μL/L 30% (v/v) $H_2O_2$ solution for 5 min at 37°.

*Protein determination.* Protein concentration was determined by the method of Lowry *et al.* [26] using bovine serum albumin as a standard.

## RESULTS AND DISCUSSION

Primary cultured rat liver parenchymal cells were exposed to 1 nM TCDD for 23 and 47 hr, and then labeled with [$^{35}$S]methionine for 1 hr. The cell lysates prepared from TCDD exposed and control cells were separated by SDS–polyacrylamide gel electrophoresis and $^{35}$S-labeled proteins were detected by fluorography. Figure 1 shows that the synthesis of two proteins with $M_r$s of 26,000 and 54,000 was increased markedly by TCDD exposure for 23 hr (lane 2). These proteins were designated 26k-P and 54k-P, respectively. Prolonged exposure to TCDD (47 hr) caused the increase in synthesis of not only 26k-P and 54k-P but an additional protein with $M_r$ of 39,000 (designated 39k-P) (lane 4). The pattern of newly synthesized protein in control cells after 47 hr was essentially identical to that after 23 hr.

Cytochromes P450IA1 and P450IA2 are major iso-proteins of cytochrome P450 induced in rat liver by TCDD [12, 27]. To determine whether 26k-P and 39k-P were identical to cytochromes P450IA1 and P450IA2 or related proteins, cytochrome P450 induced in liver parenchymal cells was identified immunochemically using western blotting [25]. Anti-rat cytochrome P450IA1 rabbit IgG, which is reactive with both cytochromes P450IA1 and P450IA2, was used for immunostaining [28]. As shown in Fig. 2, two proteins (a major protein and a minor protein with smaller $M_r$) reacting with IgG were detected in the cells exposed to TCDD for 47 hr, but were not observed in the control cells. Because the $M_r$ of cytochrome P450IA1 is higher than that of cytochrome P450IA2 [29], the upper major band and lower minor band in Fig. 2, lane 5 were identified as cytochrome P450IA1 and cytochrome P450IA2, respectively. The band of 54k-P corresponds with that of cytochrome P450IA2 and, therefore, 54k-P may be identified as cytochrome P450IA2. However, 26k-P and 39k-P did not react with the IgG indicating that these proteins were different from cytochromes P450IA1 and P450IA2. Although it was demonstrated that cytochrome P450IA1 was induced in TCDD-exposed cells, a distinct radioactive band corresponding to cytochrome P450IA1 was not observed. This may be due to the cytochrome P450IA1 band overlapping with other radioactive bands (Fig. 2).

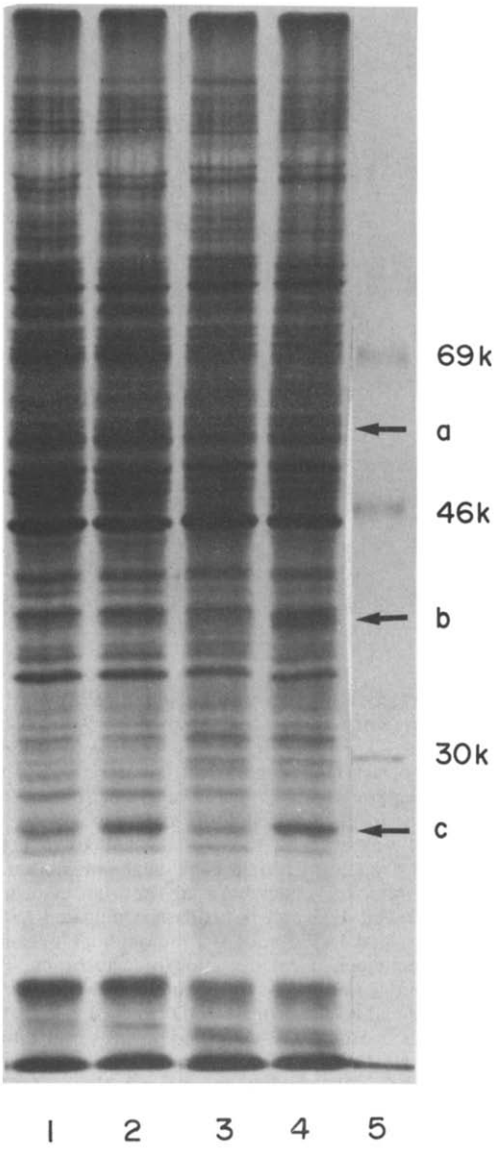


Fig. 1. SDS–polyacrylamide gel electrophoresis of $^{35}$S-labeled proteins from TCDD-exposed and control primary cultured rat liver parenchymal cells. The cells were labeled with [$^{35}$S]methionine for 1 hr after incubation with TCDD for 23 hr (lanes 1 and 2) and 47 hr (lanes 3 and 4). $^{35}$S-Labeled proteins from TCDD-exposed (lanes 2 and 4) and control (lanes 1 and 3) cells were separated on an SDS–polyacrylamide gel and detected by fluorography. Lane 5, methylated $^{14}$C-labeled proteins as $M_r$ marker: 69K, bovine serum albumin ($M_r$ 69,000); 46K, ovalbumin (46,000); 30K, carbonic anhydrase (30,000). Arrows indicate TCDD-induced proteins: (a) 54k-P; (b) 39k-P; (c) 26k-P.

In order to analyse further alterations in protein synthesis by TCDD, the cell lysate from cells exposed to TCDD for 47 hr was subjected to 2D gel electrophoresis. Synthesis of four proteins was stimulated in the TCDD-exposed cells (indicated by circles in Fig. 3A and B). Judging from their $M_r$s, the proteins in circles 1 and 2 seems to be 26k-P and

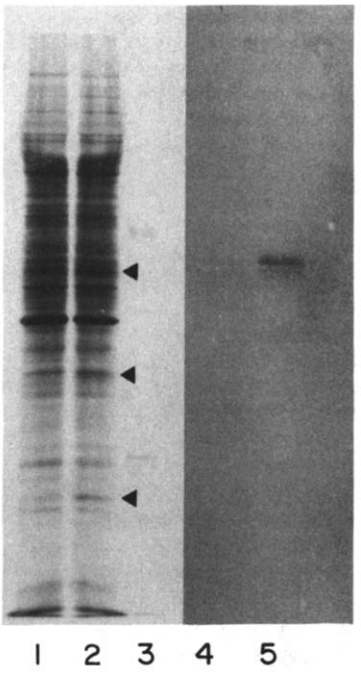


Fig. 2. Detection of cytochrome P450 in cell lysates. $^{35}$S-labeled proteins (200,000 cpm) from the cells exposed to TCDD for 47 hr and the control cells were subjected to SDS–polyacrylamide gel electrophoresis. $^{35}$S-labeled proteins separated on the gel were electrophoretically transferred to a nitrocellulose membrane. After the membrane was exposed to an X-ray film to detect the labeled proteins, cytochrome P450 on the membrane was detected immunochemically using anti-rat cytochrome P450IA1 rabbit IgG and peroxidase conjugated to anti-rabbit IgG goat IgG. Lanes 1–3, autoradiogram; lanes 4 and 5, immunochemical detection of cytochrome P450; lanes 1 and 4, the cell lysates from control cells; lanes 2 and 5, the cell lysates from TCDD-exposed cells; lane 3, $M_r$ markers, methylated $^{14}$C-labeled bovine serum albumin (top, $M_r$ 69,000) and carbonic anhydrase (bottom, $M_r$ 30,000). Arrow heads indicate the positions of 54k-P, 39k-P and 26k-P (top to bottom).

39k-P, respectively. The proteins with $M_r$ of 54,000 in circles 3 and 4 may be 54k-P (cytochrome P450IA2) and cytochrome P450IA1, respectively, since cytochrome P450IA2 is slightly smaller and more basic than cytochrome P450IA1 [29]. On the other hand, synthesis of three proteins with $M_r$ of 24,000, 25,000 and 29,000 (24k-P, 25k-P and 29k-P, respectively) was inhibited by TCDD exposure. These proteins remain to be identified.

The *in vivo* effect of TCDD on the amount of different proteins in rat liver was examined also. TCDD-treated and control rats were killed on the 14th day after TCDD administration. Specific biochemical changes, such as the diminishing of glucocorticoid receptors and elevation of glutamine synthetase activity, have been reported to occur in TCDD-treated rats on the 14th day [21]. Crude extracts were prepared from the liver tissues after homogenizing with a buffer containing NP-40 and were separated by 2D gel electrophoresis. Not only soluble proteins but also mitochondrial and microsomal proteins were solubilized effectively from the tissue by the method used here [22]. Typical data is shown in Fig. 3C and D. The amount of seven proteins (two proteins with $M_r$ of 26,000, one of 36,000, two of 39,000 and two of 54,000) were increased in the liver from a TCDD treated rat (squares in Fig. 3C).

In order to elucidate whether the rat liver proteins increased by TCDD administration were identical to the proteins stimulated *in vitro* in the liver cells, the crude extract from a TCDD-treated rat liver and $^{35}$S-labeled cell lysate from TCDD-exposed cells were co-migrated on the 2D gel. Circles in Fig. 3C indicate the positions of $^{35}$S-labeled proteins stimulated *in vitro*. TCDD treatment resulted in the induction of two proteins with $M_r$ of 54,000 both *in vivo* and *in vitro*, which appear to be 54k-P (cytochrome P450IA2) and cytochrome P450IA1 (Fig. 3C, squares 6 and 7, respectively). However, the positions of 26k-P and 39k-P from *in vitro* samples (Fig. 3C, circles a and b, respectively) were different to those observed for *in vivo* samples. This difference in migration positions may be due to a modification of charges on these proteins, which may be related to the length of exposure to TCDD. In order to elucidate modification of the proteins stimulated *in vitro*, liver parenchymal cells need to be cultured for over 1 week. In the present study, the cells could be maintained in a good condition for 4 days only [19]. Further study is under way to improve the cell culture technique.

TCDD has been shown to have a number of biological effects in addition to its carcinogenic and teratogenic activities [6, 11]. For example, it has been shown to alter the activities of epidermal growth factor [30] and steroid hormones [21, 31], and to activate protein kinase [32]. However, the mechanisms of these biological activities have yet to be elucidated. Induction of cytochrome P450 is a well-documented effect of TCDD and its mechanism has already been well examined [6, 7, 11], but all the biological effects of TCDD cannot be explained simply by the induction of cytochrome P450. Although TCDD-induced proteins other than cytochrome P450 might have important roles in the various biological activities of TCDD, the effect of TCDD on the synthesis of other proteins has not yet been studied extensively.

We demonstrate here that synthesis of two proteins (26k-P and 39k-P) other than cytochrome P450 was stimulated in primary cultured rat liver parenchymal cells by TCDD exposure. Increase in synthesis of these proteins by TCDD has not been reported previously. The presence of small amounts of 26k-P and 39k-P in the control cells indicate that the synthesis of these endogenous proteins may be amplified by TCDD. Synthesis of 26k-P and 39k-P can be stimulated either by the direct effect of TCDD or non-specific reaction to xenobiotics, such as proliferation of hepatic endoplasmic reticulum. However, synthesis of 26k-P and 39k-P was not stimulated by exposure to 2 mM phenobarbital, 20 $\mu$M *p*-aminoacetophenone and 10 $\mu$M sodium arsenite (an inducer of heat shock proteins) (unpublished data), suggesting that stimulation of

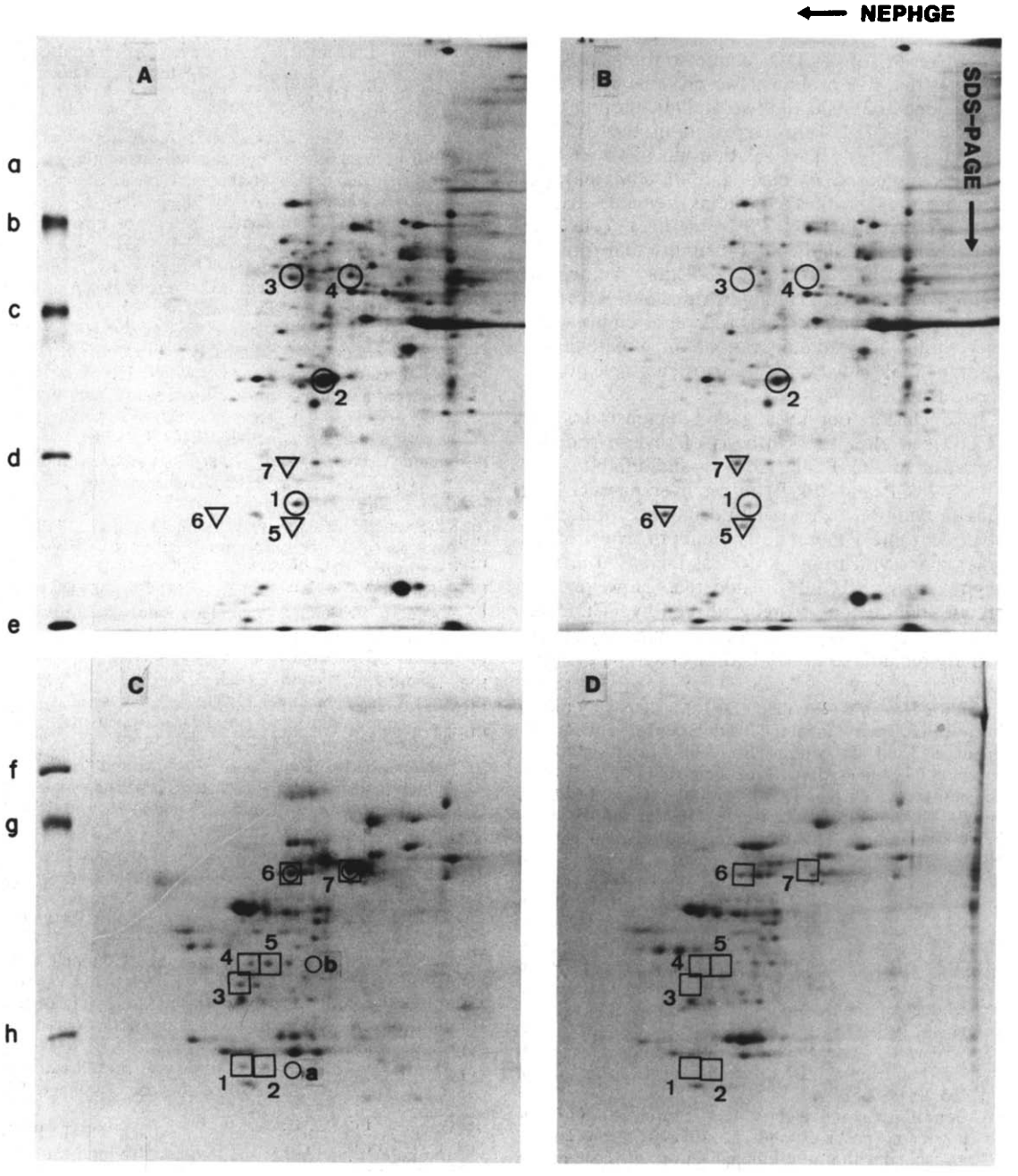


Fig. 3. 2D gel electrophoresis of $^{35}$S-labeled proteins from rat liver parenchymal cells and proteins extracted from rat liver, performed using NEPHGE for the first dimension. $^{35}$S-Labeled proteins from TCDD-exposed (47 hr exposure, panel A) and control (panel B) cells were subjected to 2D gel electrophoresis and proteins separated on the gel were detected by fluorography. Circles and triangles on panels A and B indicate the proteins whose synthesis was stimulated and inhibited by TCDD, respectively: (1) 26k-P; (2) 39k-P; (3) 54k-P (cytochrome P450IA2); (4) cytochrome P450IA1; (5) 24k-P; (6) 25k-P; (7) 29k-P. The crude extracts from a TCDD-treated (panel C) and a control (panel D) rat liver (200 $\mu$g protein each) were also subjected to 2D gel electrophoresis and proteins on the gel were stained by Coomassie Brilliant Blue R-250. Squares indicate the proteins which were increased in rat liver after TCDD administration: (1) and (2) proteins with $M_r$ of 26,000; (3) $M_r$ of 36,000; (4) and (5) $M_r$ of 39,000; (6) and (7) $M_r$ of 54,000. Circles on panel C (a, b, 6 and 7) indicate the positions corresponding to 26k-P, 39k-P, 54k-P (cytochrome P450IA2) and cytochrome P450IA1, respectively, which were determined by the co-migration of liver crude extract and $^{35}$S-labeled proteins from TCDD-exposed cells. NEPHGE and SDS indicate the direction of first and second dimensional electrophoresis, respectively. $M_r$ markers: (a) and (f) phosphorylase b ($M_r$ 97,000); (b) and (g) bovine serum albumin (69,000); (c) ovalbumin (46,000); (d) and (h) carbonic anhydrase (30,000); (e) cytochrome *c* (12,000); (a–e) methylated $^{14}$C-labeled proteins detected by fluorography; (f–h) stained by Coomassie Brilliant Blue R-250.

these proteins is not caused by non-specific reaction to xenobiotics.

The result of TCDD administration *in vivo* indicates that five proteins (two proteins with $M_r$ of 26,000, one of 36,000 and two of 39,000) other than cytochrome P450 were increased in the TCDD-treated rat liver. The relationship between the proteins increased *in vitro* and *in vivo* and the identification of these proteins remains to be revealed. Although 60–70% of liver cells are parenchymal cells [18], the possibility that some of the proteins increased *in vivo* may belong to nonparenchymal cells cannot be ruled out. Recently, induction of several proteins was reported in mouse liver, following administration of 3-methylcholanthrene [33]. However, these proteins have not yet been identified.

In conclusion, our investigation demonstrates that TCDD can alter the synthesis of several proteins (increase of 26k-P and 39k-P, and inhibition of 24k-P, 25k-P and 29k-P) in rat liver parenchymal cells in addition to the well-documented induction of cytochrome P450. The amounts of five proteins other than cytochrome P450 was increased *in vivo* in the livers of TCDD-treated rats. However, the precise identities of proteins altered by TCDD are still unknown and their relationship to TCDD toxicity remains to be studied further.

*Acknowledgements*—We thank Dr J. McKinney (National Institute of Environmental Health Science) and Drs Y. Takahashi and T. Miura (National Institute for Environmental Studies, Japan) for their kind gifts of TCDD and anti-rat cytochrome P450IA1 rabbit IgG, respectively; and Dr M. M. Lipsky (University of Maryland) for his helpful suggestion. This work was supported in part by NIH grants (HD 41022 and HD 16596) to S.R.M.

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