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DETECTION OF THE CHANGES IN PROTEIN DISTRIBUTION OF RAT PLASMA INDUCED BY CARBON TETRACHLORIDE ADMINISTRATION BY MEANS OF TWO-DIMENSIONAL ELECTROPHORESIS

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SUMMARY

The changes in rat plasma protein distribution after carbon tetrachloride administration were examined using two-dimensional electrophoresis, utilizing isoelectric focusing in polyacrylamide gel in the first dimension and pore gradient polyacrylamide gel electrophoresis in the second dimension. Drastic changes in amount of protein were observed at more than 20 spot positions including those of transferrin, Gc-globulin and low-density lipoprotein. The time course of the changes was examined, and the most drastic changes were observed at 2 days after carbon tetrachloride administration.

INTRODUCTION

The degeneration of rat liver induced by carbon tetrachloride has been studied biochemically to understand the function of liver and clinically as a model system for human medicinal poisoning. Studies on the mechanism of fatty accumulation and fall in lipoprotein formation have been reported [1—4]. However, as for the changes in plasma protein distribution which must accompany the liver damage, little is known except for a decrease in the albumin/globulin ratio and an increase in the activities of some intracellular enzymes, due to poor resolution of the analytical techniques.

Recently, we described a two-dimensional electrophoretic technique which did not employ denaturing agents, and showed that human plasma proteins

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could be resolved into about 250 spots [5, 6]. Since the technique does not employ sodium dodecyl sulfate or urea throughout the electrophoretic run, it is suited for the analysis of mixtures of soluble proteins maintaining their native physicochemical properties [7, 8] and their biological activities [9].

In the present report we show that drastic changes in the distribution of rat plasma proteins induced by carbon tetrachloride administration could be analyzed by means of the two-dimensional electrophoretic technique. The results showed that the degeneration of rat liver after carbon tetrachloride administration could be followed by examining the plasma protein distribution patterns.

MATERIALS

Reagents

Ampholines (pH 3.5—10 and pH 3.5—5) were obtained from LKB Produkter (Bromma, Sweden). Acrylamide, N,N'-methylenebisacrylamide (both special grade for electrophoresis), glycine, Tris base, and ammonium persulfate were from Wako Pure Chemical Industries (Tokyo, Japan). N,N,N',N'-Tetramethylethylenediamine and Coomassie brilliant blue R-250 (both special grade for electrophoresis) were from Nakarai Chemicals (Kyoto, Japan). Heparin was obtained from Kodama (Tokyo, Japan).

Plasma samples

Male rats (Wistar strain, 18 weeks) weighing approximately 350 g were used. Food was withdrawn 12 h before carbon tetrachloride feeding. Carbon tetrachloride, 20% (v/v) in liquid paraffin, was introduced into the stomach under light ether anesthesia by intubation at a dose of 0.5 ml per 100 g of body weight. At scheduled time intervals 5 ml of blood were taken from the descending aorta of each rat with a disposable syringe, the inside of which had been coated with heparin solution. The blood was centrifuged for 15 min at 3000 *g*. Sucrose was added to the supernatant plasma to give a concentration of 40% (w/v), and the plasma sample was stored at —20°C.

METHODS

Two-dimensional electrophoresis in the absence of denaturing agents

The technique of two-dimensional electrophoresis in the absence of denaturing agents was described previously [5, 6]. First-dimension isoelectric focusing was performed on gel columns 14 cm × 0.5 cm I.D. A 4% acrylamide (0.2% bisacrylamide) solution containing 2% Ampholine pH 3.5—10, 0.5% Ampholine pH 3.5—5, and 0.05% ammonium persulfate, was poured into a glass tube. Gelling occurred in about 20 min. The electrode solutions were 0.04 *M* NaOH (cathode) and 0.01 *M* phosphoric acid (anode). An overlay solution (2% Ampholine pH 3.5—10 and 10% sucrose, 50 μl) was layered on top of the gel columns and then plasma samples (50 μl) were applied under the overlay solution. Electrophoresis was run at 2 mA constant current for 40 min and then at 460 V constant voltage for 20 h at 3°C. After electrophoresis, a rubber bulb was placed on top of the tube and the gel was pushed out and

placed on top of the second-dimension slab gel without equilibration.

Second-dimension gradient polyacrylamide gel electrophoresis was performed with a slab gel apparatus which forms a slab gel of 12 cm long, 16 cm wide, and 0.4 cm thick. A 4—21% acrylamide linear gradient (0.2% bisacrylamide) containing a 0—10% sucrose gradient and a 0.05—0.025% ammonium persulfate gradient was poured in about 50 min at 4°C. Gelling occurred in about 2 h in a water-bath at 30°C. The gradient gel buffer was 0.14 *M* Tris·HCl (pH 8.9) and the electrode buffer was 0.05 *M* Tris—0.38 *M* glycine (pH 8.3). Electrophoresis was run at 36 mA constant current for 20 h.

Measurement of the pH gradient

The isoelectric focusing gel was duplicated for each sample; one was cut into 10-mm sections which were placed in individual vials containing 2 ml of distilled water. These vials were capped and left to stand for 2 h, then the pH was measured on a pH meter.

Staining and destaining

The gel was stained overnight in 0.025% Coomassie brilliant blue R-250—7% (v/v) acetic acid—50% (v/v) methanol, and destained in 7% acetic acid at 80°C for 4 h, then in two changes of 7% acetic acid at room temperature for two days.

Photography

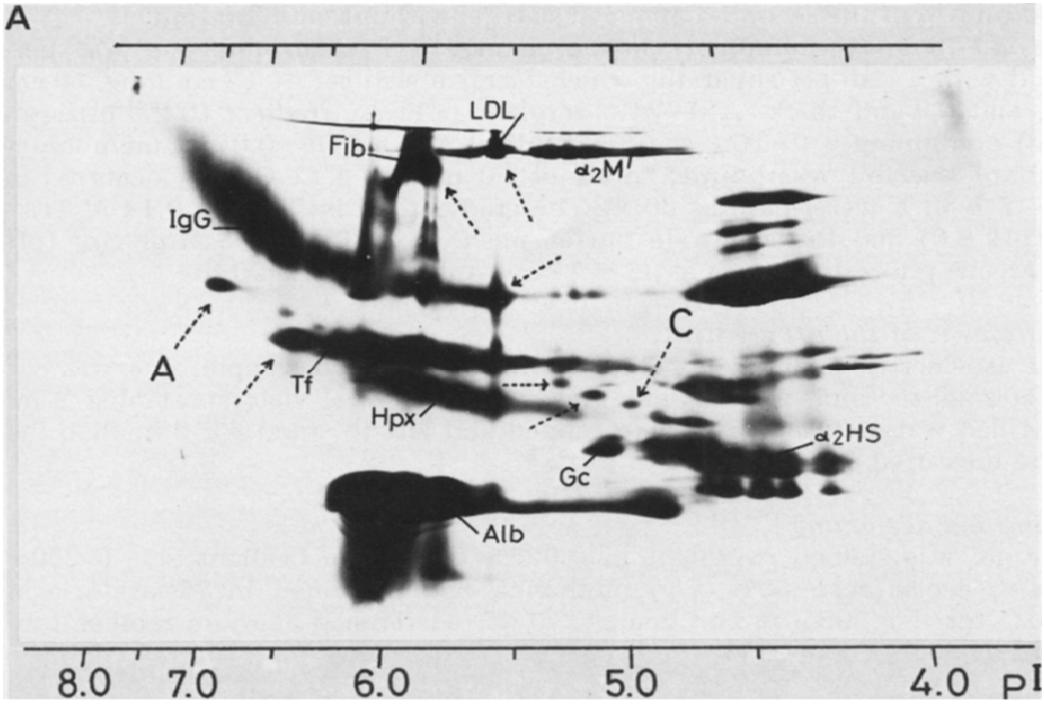
Photography was carried out by placing the gel on top of a viewing box positioned under a 35-mm reflex camera. A Toshiba Y-2 filter (Tokyo Shibaura Electric Co., Tokyo, Japan) was attached to the camera and Fuji minicopy II film (ASA 32, Fuji Photo Film Co., Tokyo, Japan) was used.

Densitometry

Densitometric quantitation of Coomassie blue-stained spots was carried out using a Shimadzu dual-wavelength thin-layer chromatographic (TLC) scanner CS-910 (Shimadzu Corp., Tokyo, Japan). Sample wavelength was set at 580 nm and reference wavelength was 750 nm. The densitometer was operated in "zig-zag scanning mode" and the protein amounts were quantitated by measuring the step height of the integrating signal.

RESULTS

Fig. 1A shows one of the two-dimensional electrophoretic patterns of normal rat plasma proteins, before administration of carbon tetrachloride. The protein distributions of several rat (Wistar strain) plasma samples were compared and the positions of the plasma proteins were quite reproducible. Major plasma proteins were located on the gel by comparing the patterns with those of human plasma proteins [6, 10]. Fig. 1B shows an example of the two-dimensional distribution of plasma proteins of a rat administered carbon tetrachloride (the plasma was taken 72 h after the administration). Drastic changes in protein distribution were observed. The locations of the proteins, the positions or spot areas of which apparently changed by the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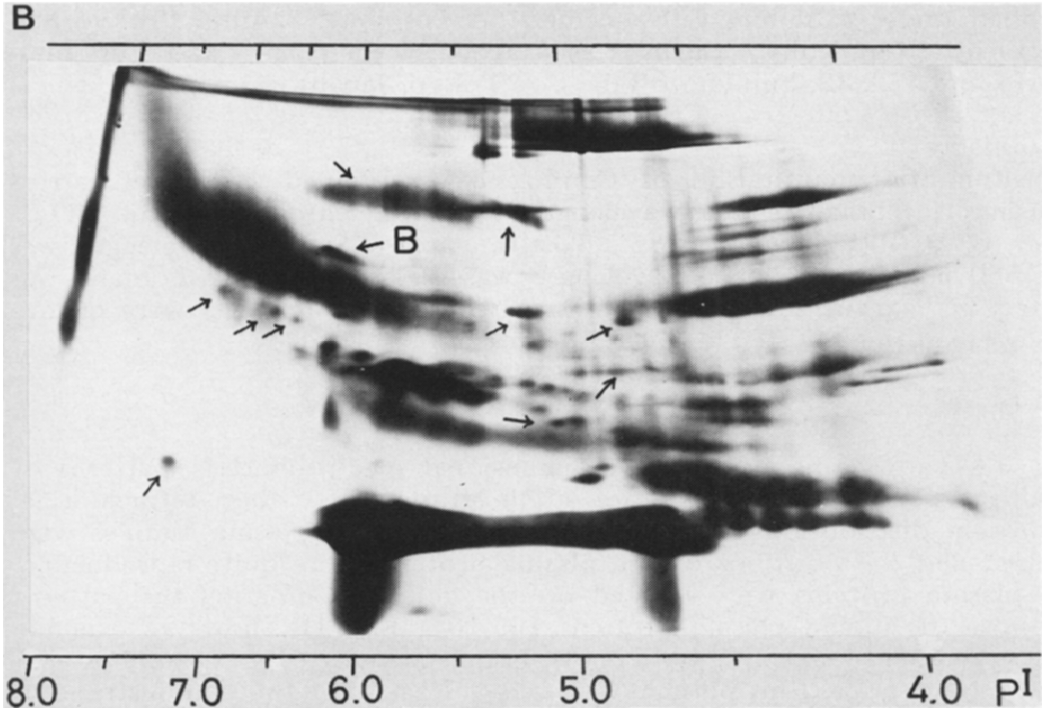
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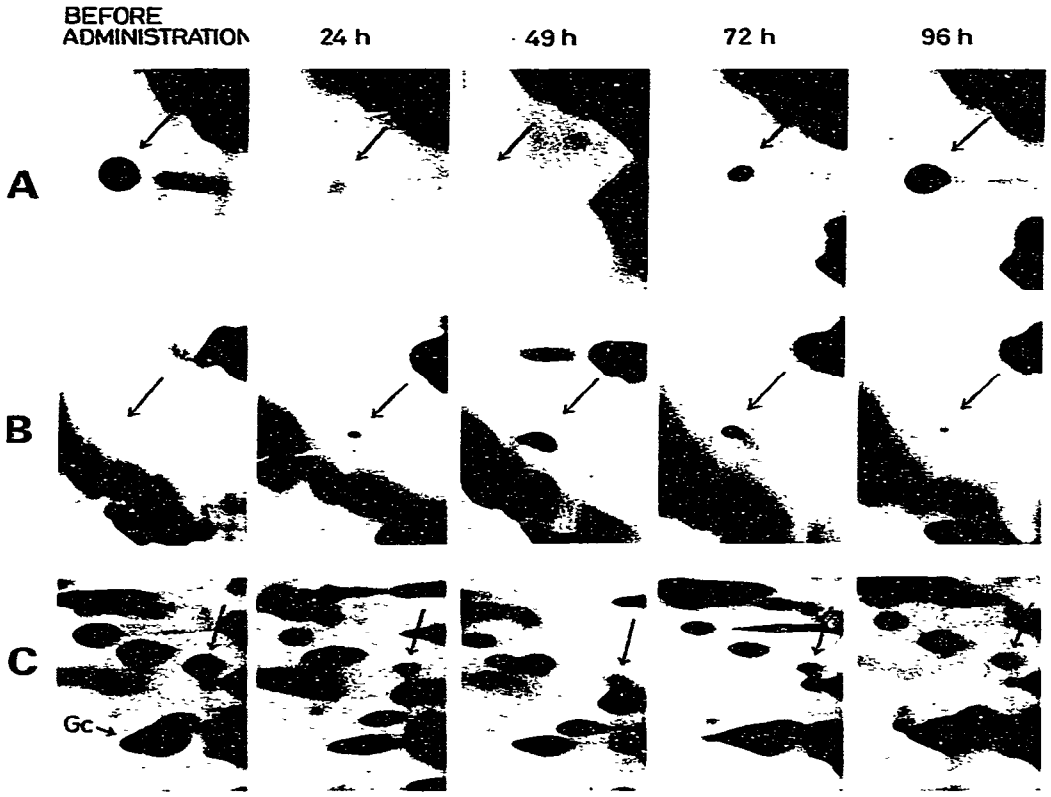

Fig. 2. Time course of the changes in the two-dimensional pattern of rat plasma proteins. A rat was administered carbon tetrachloride, and plasma samples at 0, 24, 49, 72, and 96 h after administration were analyzed by means of two-dimensional electrophoresis. Time-dependent changes at the gel sections around spot A (A), spot B (B) and spot C (C) are shown. Spot positions are indicated by arrows. Gc = Gc-globulin.

administration of carbon tetrachloride, are shown by arrows in the figure. The proteins that apparently decreased after administration are indicated by dotted arrows in Fig. 1A, and those that appeared are indicated by solid arrows in Fig. 1B. Some of the major plasma proteins, which were tentatively identified as IgG, albumin, hemopexin, and α_2HS-glycoprotein, were not affected by carbon tetrachloride administration. However, fibrinogen, transferrin, and Gc-globulin were apparently affected.

The time course of the changes in the two-dimensional pattern of plasma proteins was examined. A rat was administered carbon tetrachloride and plasma samples taken after 24, 49, 72, and 96 h were compared with plasma taken

Fig. 1. Two-dimensional electrophoresis of rat plasma proteins in the absence of denaturing agents. (A) Before administration of carbon tetrachloride, (B) 72 h after administration of carbon tetrachloride. Arrows with a dotted line in (A) indicate the spots that apparently decreased in area or disappeared after carbon tetrachloride administration. Arrows with a solid line in (B) indicate the spots that appeared after carbon tetrachloride administration. The positions of major plasma proteins were located on the gel by comparing the patterns of rat plasma proteins with those of human plasma proteins [10]. IgG = immunoglobulin G; Fib = fibrinogen; LDL = low density lipoprotein; α_2M = α_2-macroglobulin; Tf = transferrin; Hpx = hemopexin; Gc = Gc-globulin; α_2HS = α_2HS-glycoprotein; Alb = albumin.

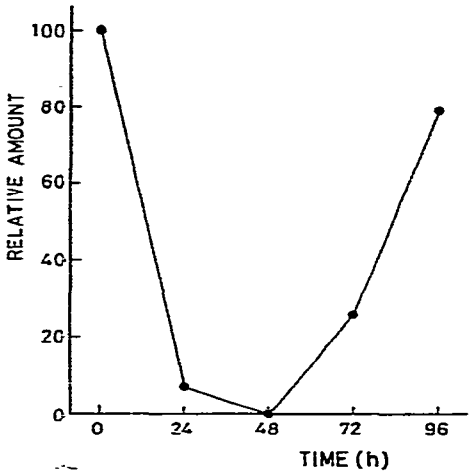

Fig. 3. The quantity of spot A after carbon tetrachloride administration as a function of time. Spots A on acrylamide slab gels were quantitated using a TLC scanner.

before administration. The time-dependent changes at the gel sections around spots A, B, and C (indicated by arrows in Fig. 1) are shown in Fig. 2, in which the positions of the three spots are indicated by arrows. The area of spot A which decreased after carbon tetrachloride administration, disappeared at 49 h after administration, then increased at 72 h, and almost recovered its original level at 96 h. In contrast, the protein of spot B appeared 24 h after administration and the spot area was maximum at 49 h, then it decreased with lapse of time. The area of spot C decreased at 24 h, but the spot did not disappear at 49 h, nor did it recover its original level at 96 h.

Densitometric quantitation of spot A was carried out with a Shimadzu CS-910 densitometer. Fig. 3 shows the time course of the quantity of spot A. A drastic fall in the amount of spot A at 49 h after carbon tetrachloride administration and its recovery at 96 h after administration were demonstrated.

DISCUSSION

One of the purposes of this study was to find out if the two-dimensional electrophoretic technique could contribute to diagnosing the progress of diseases. As an experimental disease, poisoning of rats with carbon tetrachloride was chosen since it has long been studied chemically and histologically [1—4]. As shown in Figs. 1—3 the technique can detect changes in plasma protein distribution caused by carbon tetrachloride. The time course of the increase or decrease of each protein spot also could be followed by means of the technique. The proteins affected by carbon tetrachloride administration can be divided into two types: type 1 includes proteins that disappeared (e.g. spot A in Fig. 2) or decreased in amount (e.g. spot C in Fig. 2) after carbon tetrachloride administration, and type 2 includes those that newly appeared (e.g. spot B in Fig. 2). These proteins specifically affected by carbon tetrachloride may be related to the mechanism of liver degeneration.

The electrophoretic technique employed isoelectric focusing in the first dimension and acrylamide pore gradient (4—21%) electrophoresis in the second

dimension. The technique uses no denaturing agent such as urea or sodium dodecyl sulfate throughout the run, thus equilibration of the first-dimension gel was not necessary. Further, when the isoelectric focusing gel was examined for the protein remaining after the second-dimension run, no Coomassie blue-stained band was observed. Therefore, comparison of spot areas was possible since there was no loss of proteins in the course of the electrophoretic run. Densitometric quantitation of isolated spots such as spot A was readily performed using a commercial TLC densitometer. The quantitation will help to determine the degree of poisoning.

The amounts of low-density lipoprotein, fibrinogen, transferrin and spot A decreased drastically as shown in Fig. 1A and B. Spot A showed a relatively basic p*I* (7.2) and its molecular weight was calculated to be about 110,000. From these values we suppose that spot A may be one of the complement components.

As for the spots that newly appeared after carbon tetrachloride administration (e.g. spot B), individual identification will not be easy since they can be either cellular proteins, or modified plasma proteins. Extraction of proteins from fixed, stained two-dimensional gels and amino acid micro-analysis of the extracted proteins have been performed for human plasma proteins [10]. These are promising techniques for further analysis of the proteins specifically affected by carbon tetrachloride.

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