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Effects of Aroclor 1254 on proteins of mouse liver: Application of two-dimensional electrophoretic protein mapping

Liver proteins of male C57BL/6T mice treated with 0, 50, or 250 mg/kg Aroclor 1254 were analyzed by high-resolution two-dimensional (2-D) electrophoresis. The resulting patterns were processed using a computerized image analysis system and quantitative data selected for a total of 150 protein spots. On the basis of an analysis of liver proteins from five animals in each treatment group, we found 31 proteins that showed quantitative differences attributable to treatment with chlorinated hydrocarbons at a high level of statistical significance. One of the altered proteins appears to be Mitcon:2, a heat-shock sensitive mitochondrial matrix polypeptide; another appears likely to be microsomal cytochrome b_5. The results indicate that quantitative 2-D protein mapping may reveal much more detail regarding the *in vivo* effects of toxic xenobiotics than has previously been available, and thus allow a more informative approach to the testing of toxic compounds.

1 Introduction

The thesis of the present paper is that protein mapping can provide a detailed picture of what happens in the tissues of an animal treated with a potentially toxic compound. Such an approach is possible because protein mapping allows the simultaneous examination of a large number of proteins in samples of biological interest and thereby makes possible a comprehensive approach to the problem of defining effects of experimental manipulation on gene expression. The method is based on a system of two-dimensional (2-D) electrophoresis [1–3], combining a separation by isoelectric focusing in urea and NP-40 detergent with a subsequent perpendicular separation by electrophoresis in sodium dodecyl sulfate (SDS). When used with a computerized system capable of analyzing the resulting 2-D pattern, protein mapping allows accurate quantitative measurement of the abundance of large numbers of distinct polypeptides [4]. In contrast to the use of enzyme or immunological assays, protein mapping does not depend on previously obtained knowledge about enzymatic activity or on the existence of antibodies specific for a molecule to be measured. It is primarily a tool for discovering proteins and regulatory effects.

A range of regulatory effects has been examined by protein mapping of cells treated *in vitro*. These include heat shock [5], methionine starvation [6], and treatment with steroid hormones [7], interferon [8], phorbon esters [9], and anti-mitochondrial agents [10], among others [11]. In many cases, specific proteins were discovered whose synthesis or degradation is substantially altered by treatment. These results indicate that a comprehensive and quantitative study of drug effects might reveal patterns of change specific to particular compounds, and that identifying the major responsive proteins might provide clues regarding the molecular mechanisms underlying toxic effects. Through this approach, a comprehensive "molecular pathology" might be possible, provided the technique could be successfully applied to samples obtained from treated whole animals. Work in the area of mutation detection in mice [12–14], the analysis of heat shock effects in hyperthermic rats [15] and the analysis of human muscle samples [16] has indicated the general feasibility of mapping proteins from animal tissue. In addition, Heydorn *et al.* [17], have employed quantitative measurement of proteins on 2-D gels of brain tissue to study the effects of psychoactive drugs in the rat, thereby demonstrating the identification of new proteins affected by drug treatment. Liver represents a tissue of equal, and perhaps greater, pharmacological and toxicological interest, and we have therefore chosen it as a prototype for a series of *in vivo* drug effect studies. The first objective is to test whether statistically significant quantitative changes in the abundance of liver proteins of treated mice can be detected by this method despite the variability of individual animals.

As a test case, we elected to examine mice treated with a widely-studied mixture of chlorinated biphenyls (Aroclor 1254). Such compounds are known to cause changes in the composition and activity of liver microsomes [18, 19], and a series of 2-D electrophoretic studies of purified rat microsomes by Vlasuk and co-workers [19, 20] has demonstrated that many microsomal enzymes can be resolved and identified. However, since the microsomal enzymes represent only a fraction of total cellular protein, it was not clear whether the most abundant 700–800 proteins routinely observed on Coomassie Blue-stained 2-D gels of whole liver homogenates include microsomal polypeptides. With the added capability of quantitating a large number of proteins, including many minor components, it is now possible to search systematically for effects at other subcellular sites and for effects involving smaller quantitative changes than those readily observable by eye. The possibilities for differentiating between the effects of different compounds and for discovering new sites of damage are correspondingly expanded.

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Abbreviations: 2-D, two-dimensional; **SDS**, sodium dodecyl sulfate; **CV**, coefficient of variation; **PC**, principal component

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2 Materials and methods

2.1 Treatment of mice

Groups of 5 male C57BL/6T mice received an intraperitoneal injection of Aroclor (50 or 250 mg/kg) or corn oil vehicle on days 1 and 3, and were sacrificed on day 6. The apical end of the left lateral lobe of the liver was immediately excised and homogenized in a solution consisting of 9 M urea, 2 % Nonidet P-40 detergent, 2 % mercaptoethanol, 2 % pH 9–11 carrier ampholytes (LKB) at pH 9.5. This solution is specifically designed to dissociate protein aggregates, stop the action of protein-altering enzymes, and prevent the formation of a DNA gel. Samples were then centrifuged for 30 min at 100 000 *g* to remove insoluble material, and the supernatants were stored frozen at −80 °C.

2.2 Protein mapping

One sample from each treated and control mouse was analyzed using the 8″ × 10″ version of the ISO-DALT 2-D electrophoresis system previously described [2, 3]. The ISO-DALT system can produce an extended series of superimposable gels [21] on which many proteins can be measured with coefficients of variation (CV) better than 10 % [22]. First dimension gels containing 50 % pH 5–7, and 50 % wide-range Biolyte carrier ampholytes were focused for 30 000 Volt-hours. Second dimension 9–17 % gradient SDS slab gels were prepared by using a computer-controlled device ("Angelique"), and were electrophoresed for 26 h at 12 °C. The gels were then stained overnight with Coomassie Brilliant Blue R-250 (Serva Blue R) and destained to a clear background. All gels were run as a single batch. Samples of purified rat liver cytochrome b_5, NADPH cytochrome reductase, and a series of cytochromes P-450 were made available by Dr. James Hardwick, also of this laboratory. Electrophoresis of the pure proteins was performed individually and in conjunction with rat and mouse liver samples.

2.3 Computer analysis

Gels were scanned at 100 micron resolution through a green filter by using an Eikonix 785 operating at 12 bits grey-scale range (converted to 8-bit optical densities). The digitized images (2048 × 2048 pixels) were processed using the TYCHO system [4] to yield lists of spot positions and abundances. Gels were matched together using the GR42 software system (constructed by Dr. J. Taylor of this laboratory), and a set of 150 spots present and well-measured on all gels [23] was selected for statistical analysis [24]. The spots were given experiment-specific serial numbers; a permanent spot numbering system and associated nomenclature for mouse liver are presently under construction. Data were scaled by setting the sum of all selected spots equal for all gels. Principal component (PC) analysis was performed by using the ARTHUR software package (Infometrix Corp.) on a VAX 11/780 computer, and used the 31 spots showing most significant differences among control and treated groups by F-test ($P < 0.001$). Using a more stringent modified t-test (assuming unequal variances and only four degrees of freedom) to examine differences between control and high-dose groups, similar results were obtained (though at reduced levels of significance). The coefficient of variation for these spots within treatment groups (*i. e.* the between-animal CV) averaged 13 %. These data were scaled to give mean = 0 and variance = 1 for each variable prior to PC analysis. Analysis of variance and generation of graphs was carried out on an IBM PC/XT by using Lotus 1-2-3 (Lotus Development Corp.).

3 Results

The 2-D electrophoretic pattern of C57BL/6T mouse liver (Fig. 1A) consists of 500 to more than 800 resolved protein spots, depending on the protein loading and the staining method used. Analogous patterns from Aroclor-treated mice display several prominent changes at the higher dose used (250 mg/kg; Fig. 1B) that are easily detected on visual inspection (spots 37, 56, and 169). Statistical analysis revealed, however, that the visually observed changes represent only a small part of the effect (Fig. 2). More than 20 % of the proteins included in the statistical analysis (31/150) showed a change due to treatment that was significant at a level of $P < 0.001$ as measured by a one-tailed F-test in a conventional analysis of variance (ANOVA). Most of these proteins are altered by factors less than threefold, and are thus somewhat difficult to pick out by eye in comparisons of a few gels. Additional proteins showed treatment-related changes at lower levels of significance (21 proteins between $P < 0.001$ and $P < 0.01$, and 19 between $P < 0.01$ and $P < 0.05$). A total of 79 of the 150 proteins examined, including actin (a major constitutive cytoskeletal component) and serum albumin (derived from plasma contained in the tissue sample), were not significantly altered in abundance at either treatment level.

One protein (#7, Fig. 2) found to be significantly reduced in amount, though by less than 50 %, is a major mitochondrial matrix protein analogous to Mitcon: 2 of human cells [10]. An adjacent small protein spot (#30, Fig. 2) also is reduced in a similar manner and may represent a charge-modified version of the same protein. Mitcon: 2 responds to heat shock [5] and is quite variable in its level of expression among a series of different human cell types.

Protein # 56 (Fig. 2) has been identified provisionally as the microsomal cytochrome b_5 on the basis of proximity to the gel position of the purified rat liver protein and appropriate molecular mass. This protein shows the largest treatment-induced increase observed among the polypeptides examined (about fourfold). A minor treatment-induced protein of approximately 70 000 daltons (small rectangle below and to the right of spot #64, Fig. 2) is similar in position and shape (slightly elongated in the focusing dimension) to purified rat microsomal NADPH cytochrome reductase. It is induced approximately threefold by the high-dose treatment. All of the purified rat cytochromes P-450 analyzed appear, however, to be either too basic or too hydrophobic to appear on gel patterns of the type presented here (several could be observed using non-equilibrium (BASO) gel techniques; data not shown). Hence we believe that none of the protein effects we observed are likely to correspond to the classical cytochrome P-450 induction associated with Aroclor treatment.

For each of the 31 proteins showing differences at a high level of significance, the amount of protein measured in the lower dosage animals was between the control and high-dose levels to within experimental error (Fig. 2, side panel showing most significant examples). Thus treatment with Aroclor gives rise (within expected error) to an effect related to dose. Because the effects were smaller at the lower dose, the statistical

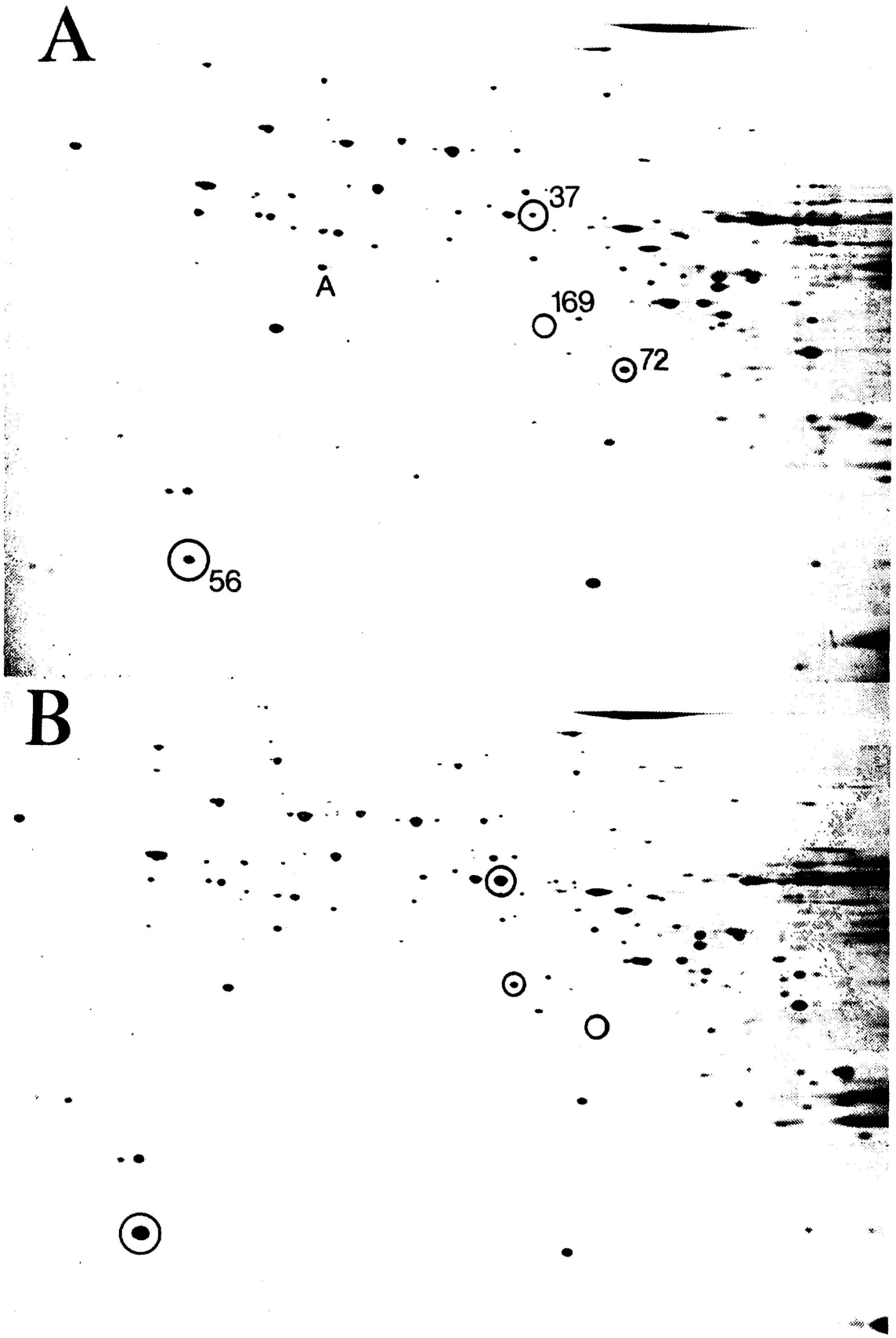

Figure 1. Coomassie Brilliant Blue-stained 2-D gels of proteins from the livers of male C57 mice either (A) untreated or (B) treated with 250 mg/kg of Aroclor 1254 as described in Section 2.1. The predominant changes observable by eye are indicated by circles, with the numbers associated with these spots in this experiment. The spots labeled A are the cellular actins, and spot #56 is likely to be cytochrome b_5.

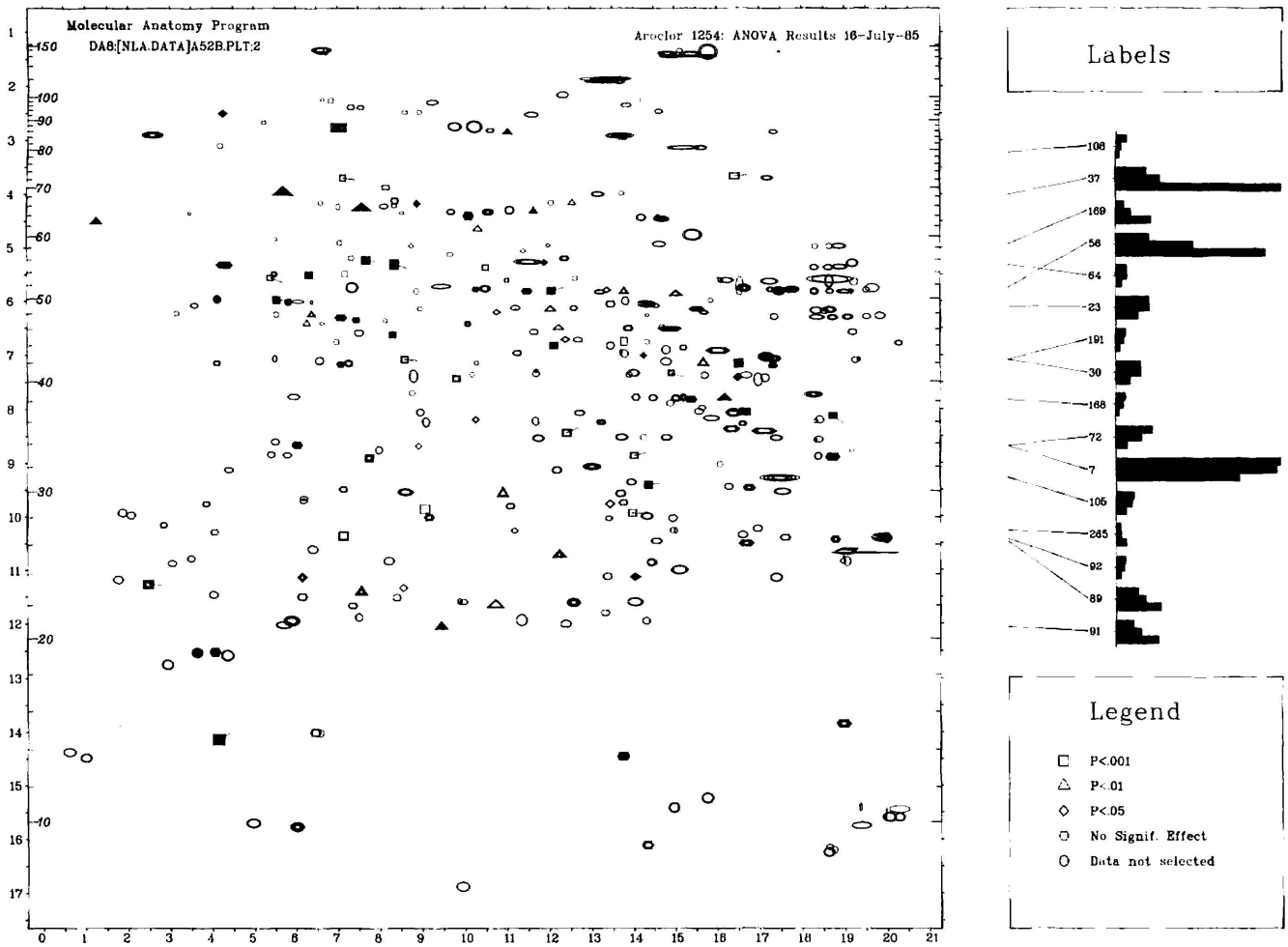

Figure 2. Schematic diagram of the C57 mouse liver pattern showing the location of proteins found to change significantly upon Aroclor treatment. Individual protein spots are plotted as symbols indicating whether they were included in the statistical analysis, and if so, their level of statistical significance. The number of contours drawn for a spot (its 'darkness') indicates its abundance in the untreated control liver. The right-hand panel identifies proteins showing most significant treatment-induced changes (lines extend from each label which, when followed with a straightedge, join with a short line segment emanating from the spot indicated), and also shows the mean levels of each protein in control, 50 mg/kg, and 250 mg/kg animals (top, middle, and lower bars, respectively, of the three-bar chart adjacent to each protein label). The scales outside the 2-D plot show distance (in cm) on the original master gel, and the vertical scale inside the plot border shows approximate SDS molecular weight derived from a series of 18 standards ([25]; Mark II).

significance was less; only three proteins showed differences significant at $P<0.001$ between control and low-dose groups. Nevertheless, the measurements indicate broad consistency with the expected dose-response relationship. As shown in Fig. 3, the abundance of most of the 31 proteins in low-dose animals is clustered between control and 50 % of high-dose levels. This result provides additional support for the belief that the observed effects are treatment-induced.

Although the reduced size and statistical significance of changes due to low-dose treatment make them difficult to detect on visual inspection of the gels, a multivariate statistical approach reveals clear separation of control and low-dose patterns (Fig. 4). A single principal component accounts for 84 % of the variation observed in the 31 spots selected as most significant and, along this component (the x-axis of the figure), the three treatment groups are clearly separated. The absence of other significant components in the pattern of change demonstrates the qualitative similarity, except for a scale factor, of the response to high and low doses of the test compound. It appears probable that the effects of a 20 mg/kg dose (less than half our low dose) could be detected by using these markers; perhaps smaller doses could be detected if more animals were used in each test group or if the exposure period were extended.

4 Discussion

In the present study, we have found at least 31 protein markers responsive to liver damage by Aroclor 1254 at a high level of statistical significance ($P<0.001$). This number represents substantially more proteins than have been observed to respond in visually interpreted 2-D gels of rat microsomes alone [19]. Hence it is unlikely that most of these proteins are associated with known components of the microsomal detoxification apparatus; indeed, the principal characterized protein expected to be induced by Aroclor treatment (cytochrome P-450) is apparently too basic to be resolved on these gels. The major protein induced by Aroclor among those studied here has been tentatively identified as cytochrome b_5 (spot #56), a well-characterized microsomal electron-transport protein. Another induced protein shares many characteristics with microsomal NADPH cytochrome reductase. In contrast, a mitochondrial matrix polypeptide (#7 in Fig. 2) appears to be decreased by treatment. It seems probable that a broad range

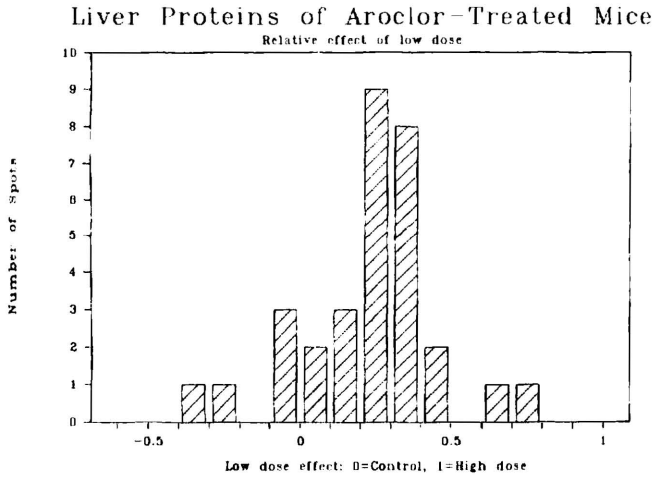

Figure 3. Low-dose effects in comparison with control and high-dose effects. The histogram, which includes only the proteins found to be significantly different between control and high-dose treatments, shows the mean amount of protein present in the five low-dose livers relative to the mean of the five control levels (positioned at 0) and the mean of the five high-dose levels (positioned at 1). Most proteins show a change at low-dose level near one-third the change associated with high-dose treatment.

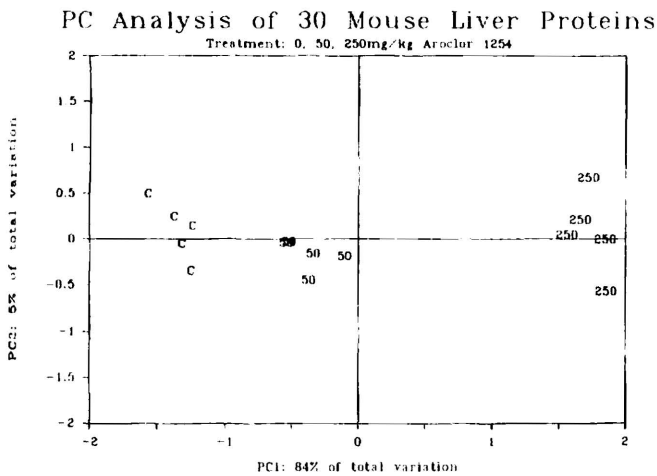

Figure 4. Plot of positions of individual mouse results in a principal component space. The horizontal dimension corresponds to a single pattern of change in the 31 treatment-related spots which accounts for 85 % of the total variance in the data. The vertical dimension corresponds to the next largest orthogonal pattern of change, accounting for a further 5 % of the variance. Each symbol indicates the position of the liver protein pattern of an individual mouse: C indicates control, and 50 and 250 indicate the respective dose levels of Aroclor. By interpreting the Aroclor-induced changes as a pattern, we can completely separate both high and low-dose livers from the controls.

of cellular proteins are affected by intoxication of this type. Although many of the alterations detected are small (two to threefold) compared to those of some major inducible enzymes, they are all of a size sufficient, if the proteins are enzymes, to cause substantial change in the behavior of metabolic pathways. Each of the markers found may represent an important part of the mechanism of toxicity; thus a detailed study of cellular localization and an attempt to discover function is warranted. A first step in this direction will be to assign each of the proteins resolved in whole liver samples to a particular subcellular compartment by means of mapping the classical cell fractions. This project is now under way.

If, as it seems likely, compounds of other toxicological classes produce different and characteristic patterns of protein change in mouse liver, then an analysis of the kind we present could be generally useful in molecular toxicology. New populations of marker proteins can be systematically discovered, and using the recently devised protein microsequencing procedures the sequences of these molecules could be determined and their genes retrieved from genomic libraries. In addition, the quantitative measurement of large numbers of markers could make possible a new level of molecular fingerprinting of drug effects. Through the use of multivariate statistical methods, the similarities and differences between drugs of similar structure could be investigated in much more detail than is currently possible.

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