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Two-Dimensional Gel Electrophoresis Analysis of the Response of *Pseudomonas putida* KT2442 to 2-Chlorophenol

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The effects of exposure of *Pseudomonas putida* KT2442 to 2-chlorophenol as a model for the chemical stress response were examined by two-dimensional polyacrylamide gel electrophoresis. Individual protein concentrations were determined at 45, 65, and 95 min following the addition of 2-chlorophenol at a concentration of 1.63 mM to exponentially growing cultures of *P. putida* KT2442 by silver staining the separated proteins. The changes in the protein concentrations could be classified into four categories, namely those which increased continuously during exposure, those which decreased in concentration, those which showed a concentration peak at some point following exposure, and those which were essentially unaffected. Thirty proteins with isoelectric points between pH 4 and 6 increased in concentration, 27 decreased, and 90 had a concentration maximum or minimum between 45 and 95 min. Of those proteins with isoelectric points between 5.5 and 10, 68 increased in concentration, 39 decreased in concentration, and 47 showed a concentration peak in the middle of the sampling period. Thus, in the evaluation of the stress response, a functional description requires an understanding both of proteins which are required at higher concentrations and of those whose presence appears to be no longer essential.

Stress proteins are expressed in response to a wide range of stress conditions. The most widely studied and best characterized of these responses is the heat shock response in various bacteria (8, 25, 30) but predominantly in *Escherichia coli* (7, 11). In nature, there are numerous other conditions under which bacteria are under stress. Such conditions include nutrient limitation, exposure to UV light, nutrient starvation, and exposure to inhibitory or toxic substrates (6, 12, 16, 18, 31, 32). The typical response involves altered patterns of gene expression and, in some cases, synthesis of stress-specific proteins. In their natural environments, bacteria are constantly exposed to conditions under which stress protein induction has been demonstrated in the laboratory. The regulation of these stress stimulons is therefore central to the activity and survival of the microorganisms. Considerable information is known concerning the regulation of the multiple operons involved in the responses to, for example, heat stress, irradiation, and nutrient limitation (33).

Since the natural environment has become a sink for many industrial, agricultural, and household pollutants, microorganisms are now also exposed to a variety of different forms of chemical stress. Early work by VanBogelen et al. (27) has shown that a considerable number of stress proteins are induced as a result of exposure to certain chemicals. They examined the effects of the chemicals ethanol, $CdCl_2$, H_2O_2, and 6-amino-7-chloro-5,8-dioxoquinoline (ACDQ) on *E. coli* and were able to show enhanced synthesis of a number of proteins. Similar results have also been shown by Ödberg-Ferragut et al. (23), Faber et al. (5), and Blom et al. (3). Unfortunately, the nature and therefore the functions of the induced proteins remain largely unknown. However, many of the chemically induced proteins which have been identified have been shown to overlap with proteins from different regulons. Gage and Neidhardt (6) found that, following exposure of *E. coli* to low concentrations of 2,4-dinitrophenol, all except three of the heat shock proteins were expressed at elevated levels. In addition, 9 of the ca. 50 proteins of the nitrogen starvation stimulon were overexpressed, as were some proteins normally enhanced under conditions of phosphorus or carbon starvation.

In the study of the stress response, one of the main tools has been the use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). By using a labelled amino acid substrate, the newly synthesized proteins can be resolved on large-format gels. Use of this technique enables the detection of the global response to a particular challenge (28). Although this technique is potentially very powerful, there have been problems associated with its use, especially with respect to the effect of cathode drift in first-dimension isoelectric focusing (24) and

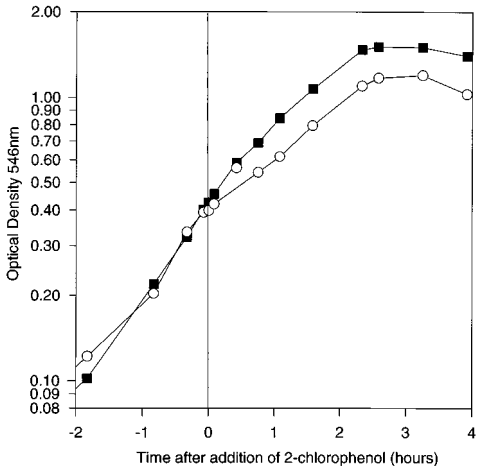

FIG. 1. Growth of *P. putida* KT2442 in the presence (○) and absence (■) of 1.63 mM 2-chlorophenol. 2-Chlorophenol was added at time zero, and growth was monitored by determining the OD_{546}. Cultures were grown in a modified Evans' minimal medium (4) at 30°C with acetate (0.15%) as the carbon and energy source.

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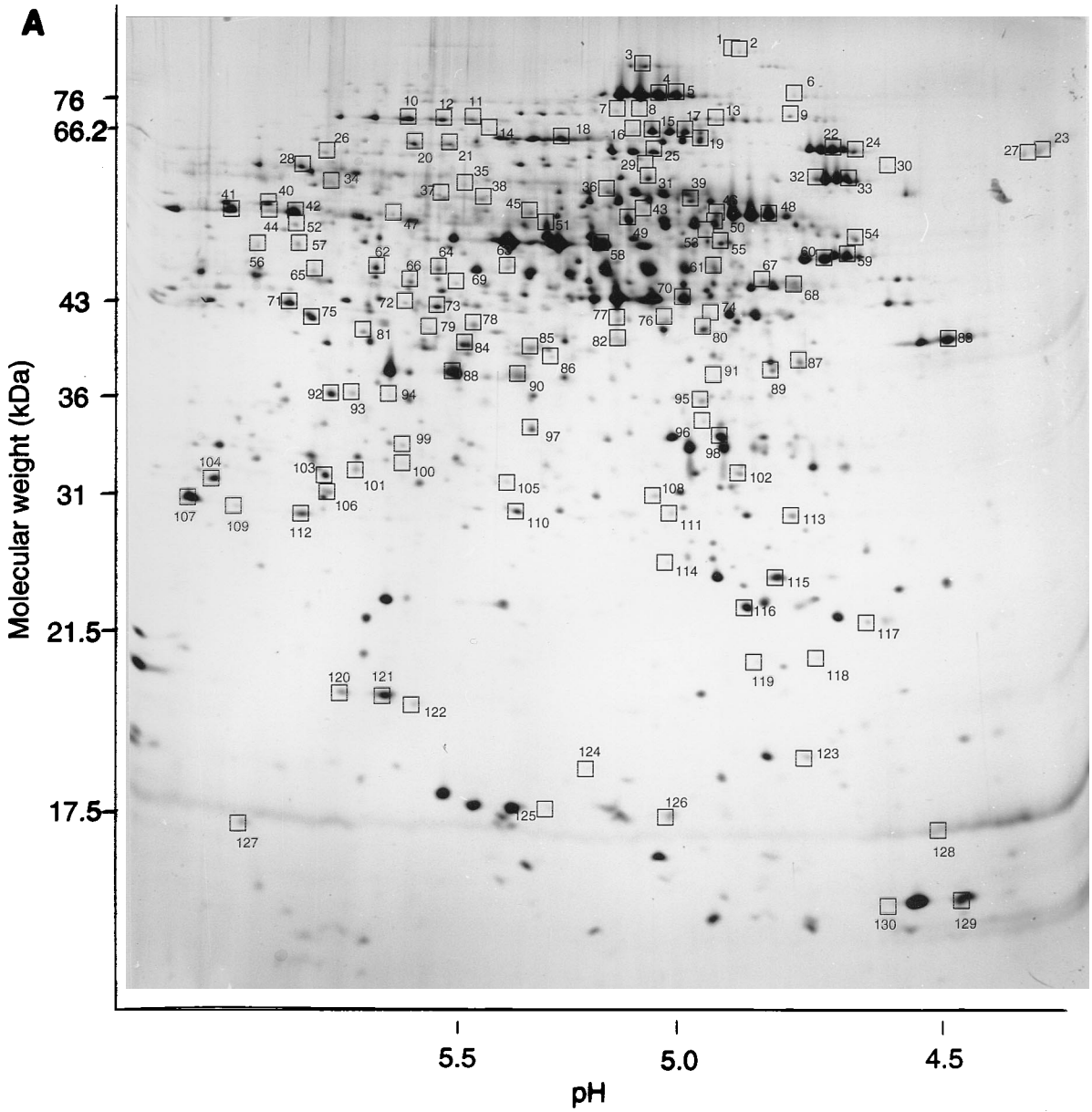

FIG. 2. Silver-stained 2D-PAGE of protein extracts of *P. putida* KT2442 from exponentially growing cells 65 min after exposure to 1.63 mM 2-chlorophenol (A) and from unexposed control cells in a parallel culture at the same point in time (B). All proteins are shown with isoelectric points in the range of pH 4 to 6. In panel A, those proteins which were present at concentrations of more than either a threefold excess or deficit compared with the corresponding protein concentrations in the untreated controls at at least one of the sampling times (45, 65, or 95 min after exposure) are boxed and numbered and quantified in Fig. 3. First-dimension isoelectric focusing was carried out by using linear Immobiline-fixed gradient gels. Second-dimension PAGE was carried out with linear gradient gels (11.6 to 17.4%).

with respect to resolution with silver staining. The latter detection method is used as an alternative to the radiolabelling technique to quantify actual protein levels. Recent innovations in isoelectric focusing, particularly the introduction of immobilized pH gradient gels (9), as well as alternative silver-staining protocols (2) have allowed considerable improvement in the application of this technique. As a consequence, in this research we were interested in using these innovations to examine changes in the actual individual protein concentrations and not in the rates of synthesis following a stress event.

Until recently, most work on stress proteins was carried out on the bacterium *E. coli*. However, for extrapolation to environmental behavior, it is necessary to understand the responses in a diverse range of different bacteria. Some limited work has been carried out on the heat shock and starvation stress responses in *Bacillus subtilis* (30) and *Pseudomonas putida* (8), but thus far little is known regarding the chemically induced stress response in such microorganisms. In the case of the heat stress proteins and starvation-induced stress proteins, some similarities have been seen, but considerably more data are necessary to provide a detailed understanding of the stress response. In this paper, we report on the response of *P. putida* KT2442 to exposure to the chemical 2-chlorophenol.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and exposure to stress. *P. putida* KT2442 was used in these studies. It was routinely maintained on Luria-Bertani agar. The

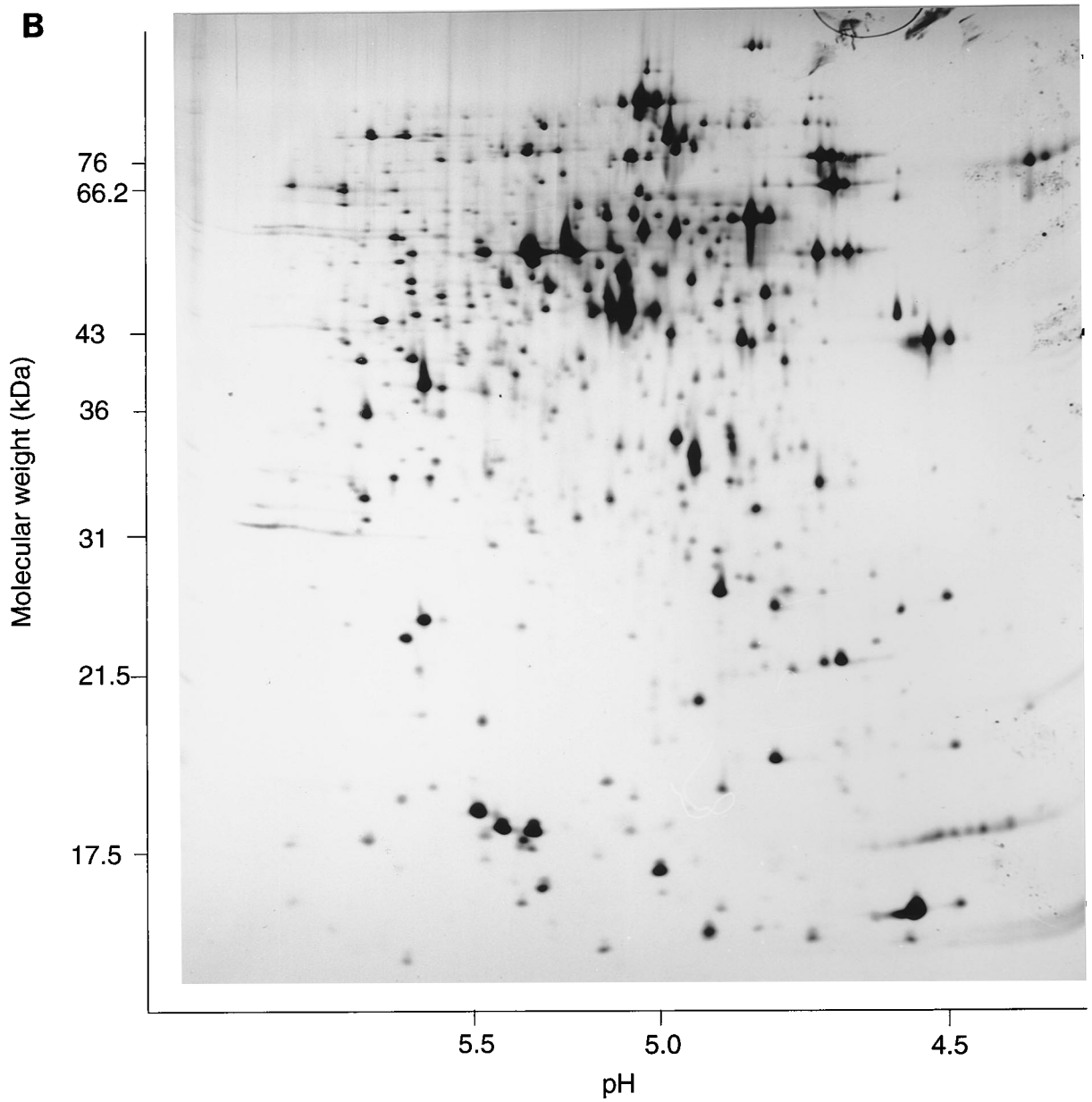

FIG. 2—*Continued.*

medium used for growth and stress studies was a modified medium based on that described by Evans et al. (4). It contained, per liter, $NaH_2PO_4 \cdot 2H_2O$, 312 mg; $K_2HPO_4 \cdot 3H_2O$, 2.28 g; NH_4Cl, 2.14 g; KCl, 298 mg; Na_2SO_4, 113 mg; $MgCl_2 \cdot 6H_2O$, 35.6 mg; $CaCl_2 \cdot 2H_2O$, 1.18 mg; $Na_2EDTA \cdot 2H_2O$, 29.3 mg; $Na_2MoO_4 \cdot 2H_2O$, 9.7 μg; ZnO, 816 μg; $FeCl_3 \cdot 6H_2O$, 10.8 mg; $MnCl_2 \cdot 4H_2O$, 4 mg; $CuCl_2 \cdot 2H_2O$, 340 μg; $CoCl_2 \cdot 6H_2O$, 952 μg; and H_3BO_3, 124 μg. Acetate (0.15% as sodium acetate) was used as the sole carbon and energy source. The pH was 7.15. Growth was carried out in 200-ml volumes in 1-liter Erlenmeyer flasks on a rotary shaker at 200 rpm. The growth temperature was 30°C. Growth was measured spectrophotometrically as the optical density at 546 nm (OD_{546}). For stress experiments, once the culture had reached early exponential phase (OD_{546} = 0.4), appropriate amounts of 2-chlorophenol were added directly to the growth flask.

Protein sampling and extraction. At appropriate times prior to and after the addition of 2-chlorophenol, 1-ml samples were taken from the culture and centrifuged (15,000 × *g*, 3 min). The pellet was resuspended in 8 μl of lysis solution (10% 0.5 M Tris-HCl [pH 6.8], 17% sodium dodecyl sulfate [15%], 5% glycerine, 8% β-mercaptoethanol). After incubation at 100°C for 4 min followed by incubation for 1 h at 37°C, 100 μl of lysis buffer (9.5 M urea, 2% Nonidet P-40, 5% β-mercaptoethanol) was added, and the sample was frozen in liquid nitrogen prior to storage at −80°C.

Determination of protein concentration. Prior to 2D-PAGE analysis it is necessary to determine the concentration of protein in the samples so that the same amount can be applied onto the gels. The sample was diluted 200 times in water for analysis. The determination of protein concentration was based on the method proposed by Kalb and Bernlohr (14), by which the amount of protein per milliliter was calculated on the basis of the absorption at 230 and 260 nm. However, since the lysis and buffer solutions absorbed strongly in the same region, correction factors had to be introduced into the original equation proposed. Therefore, the following relationship was used in this study: protein in sample (μg/ml) = $183(A_{230} - 0.48) - 75.8(A_{260} - 0.059)$.

2D-PAGE analysis of *P. putida* proteins. 2D-PAGE was carried out by using a method based on that described by Görg et al. (10) and Bjellqvist et al. (2). Protein (45 μg) was mixed with 150 μl of loading buffer {1% (wt/vol) dithioerythritol, 4% (wt/vol) 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), 54% (wt/vol) urea, 0.24% (wt/vol) Tris, 0.005% bromophenol blue solution (0.5%)}. For first-dimension isoelectric focusing, immobilized pH gradient (IPG) gels from Pharmacia LKB were used. Both linear gradient

TABLE 1. Number of proteins with isoelectric points between pH 4 and 6 whose relative concentrations changed as a result of addition of 2-chlorophenol

Sampling time (min)[a]	No. of proteins with increased concn in[b]:		No. of proteins found only in[c]:	
	Treated cells	Untreated cells	Treated cells	Untreated cells
45	18	11	5	6
65	61	43	23	9
95	24	32	11	19

[a] Time after the addition of 2-chlorophenol.
[b] Concentrations that were more than three times higher than those in the corresponding untreated or treated cells.
[c] In order to ensure those proteins which were present at high levels on one gel only and to avoid considering all of those proteins present because of variations in staining or scanning efficiency, only those proteins which contributed more than 0.1% of the total protein concentration on each gel were considered.

gels in the range of pH 4 to 7 and nonlinear IPG gels in the range of pH 3.5 to 10 were used. Before the addition of the sample, the gels were rehydrated as described by Bjellqvist et al. (2). Isoelectric focusing was carried out by using a Multiphor electrophoresis unit (Pharmacia). The proteins were separated by using a timed voltage program controlled by a Q+S Control AG, DC isolation tester 5000 NP unit. The voltage was increased from 300 to 3,000 V over 3 h, after which it was increased to 5,000 V for a total period of 90,000 V · h. Subsequent equilibration of the gels were carried out according to Bjellqvist et al. (2). 2D-PAGE was carried out by using gradient gels containing light acrylamide (25% Tris-HCl [1.5 M, pH 8.8], 37.8% acrylamide-piperazine diacrylamide (PDA) [30%:8%], 1.3% Na_2SO_4 [0.5%]) and heavy acrylamide (25% Tris-HCl [1.5 M, pH 8.8], 56.3% acrylamide-PDA [30%:8%], 1.3% Na_2SO_4 [0.5%]). The gels were poured by using a gradient mixer in a multi-casting chamber (Bio-Rad), with which up to 10 gels could be poured simultaneously to ensure identical gradients. The equilibrated strips were placed on the top of the PAGE gels and embedded in 0.5% agarose preheated to 70°C.

Proteins were separated in a Protean II electrophoresis unit (model xi for two gels and Multi-cell for six gels; Bio-Rad). The proteins were visualized by the silver-staining protocol described by Bjellqvist et al. (2). The gels were scanned by using a Molecular Dynamics laser densitometer, and the data were transferred to a Sun workstation for further analysis. Molecular weight and isoelectric point calibrations were carried out by adding 2.5 μl of protein standards from Bio-Rad to each IPG gel. Data analysis and gel evaluation were carried out by using the program MELANIE (1).

Determination of 2-chlorophenol concentration. 2-Chlorophenol was measured by high-pressure liquid chromatography (HPLC). The samples (4 ml) were centrifuged at 10,000 × *g* for 10 min, after which 3 ml of the supernatant was removed to glass vials and stored at 4°C. The vials were placed for 10 min in an ultrasound bath to remove any precipitates, 36 μl was removed to an Eppendorf tube, and 864 μl of water was added. Samples (800 μl) were added to 800 μl of buffer (4.5% orthophosphoric acid [85%], 0.378% $NH_4H_2PO_4$ [pH 2.9]). The samples were measured on a JASCO 880-PU HPLC by using a Merck RP-8 column (125 by 4 mm). The mobile phase was a methanol-H_2O mixture (1:1) degassed with helium. The detector was a JASCO 875-UV intelligent UV/VIS detector, and measurements were carried out at 220 nm.

RESULTS

Effect of 2-chlorophenol on growth of *P. putida*. The growth of *P. putida* KT2442 was inhibited by the addition of 2-chlorophenol. The extent of growth inhibition by 2-chlorophenol added at a concentration of 1.63 mM to an exponentially growing culture once it had reached an OD_{546} of 0.4 is illustrated in Fig. 1. In similar experiments when lower concentrations of 2-chlorophenol were added, the effect on growth inhibition was less pronounced (data not shown). The very high OD_{546} value measured at 25 min in the treated culture was most likely an error since in similar experiments this effect was never seen. The addition of chlorophenol to a concentration of 1.63 mM resulted in a reduction of the maximum specific growth rate by almost 24% from 0.53 to 0.41 h^{-1}.

2D-PAGE analysis of effect of 2-chlorophenol on proteins with isoelectric points between pH 4 and 6. To examine how the growth rate reduction described above was reflected in compositional changes in the proteins of *P. putida* KT2442, the experiment was repeated with samples taken at 45, 65, and 95 min after the addition of 2-chlorophenol to a concentration of 1.63 mM for separation and quantitative analysis by 2D-PAGE. Examples of the resulting silver-stained protein patterns are shown in Fig. 2 for the sample taken at 65 min along with the corresponding control gel for proteins taken from a parallel culture at the same time point without the addition of 2-chlorophenol. All *P. putida* KT2442 proteins with isoelectric points between ca. 4 and 6 have been separated in these gels. The proteins resolved on 2D-PAGE gels from the samples taken at 45 min and 95 min are not shown. In Fig. 2, there is clearly a substantial loss in resolution quality because of the need to reproduce the gels as printed photographs. However, all measurements and gel scanning were carried out with the original wet gels.

For proteins with isoelectric points between ca. 4 and 6, some variation between treated and untreated cells could be determined in the protein composition and in the amounts of the individual proteins. Table 1 summarizes the changes in the concentrations of individual proteins following 2-chlorophenol treatment and of the same proteins left untreated at the various sampling times examined. The protein concentrations were determined by laser scanning densitometry and image analysis using the computer program MELANIE (1). The largest number of changes could be seen in the samples taken at 65 min (Fig. 2).

One important implication of these results is that in the study of stress effects it is equally important to consider not only the proteins whose concentrations increase as a result of the stress (e.g., the presence of 2-chlorophenol) but also those whose concentrations decrease. To provide an overview of the changes which occurred for each of the individual proteins in Table 1, a comparison relating their concentrations at each of the other sampling times is shown in Fig. 3. The program MELANIE allows expression of the concentration of each protein as a percentage of the total protein concentration measured on the scanned image of the gel. This is the value which was used to compare one gel with the other, thereby eliminating possible differences caused by minor errors in sample application levels. For simplification, we will use the term ϕ, where ϕ is defined as follows:

$$\phi = \frac{\dfrac{\text{concentration of protein A in treated gel}}{\text{total concentration of proteins in treated gel}}}{\dfrac{\text{concentration of protein A in untreated control gel}}{\text{total concentration of proteins in untreated control gel}}}$$

Thus, ϕ describes the ratio of the protein concentration (relative to the total protein concentration measured on the gel) for individual proteins in the treated sample to the relative concentration of the same protein in the control (untreated) samples. A value of 3 indicates that the protein had a threefold higher relative concentration in cells treated with 2-chlorophenol than that found in the control. Figure 3 shows each protein which was found to have a value of ϕ in at least one of the sampling points (45, 65 or 95 min after the addition of 2-chlorophenol) in excess of ± 3 and the corresponding value of ϕ at the other two sampling times. Values for ϕ in the range of $-3 < \phi < +3$ are indicative of no significant difference.

In several cases in Fig. 3, bars are not shown for a specific protein at a particular point in time. This implies that in these cases there was no comparable protein found on either the control or treated gel at the particular sampling time. An arrow is used to indicate those instances when a protein which was

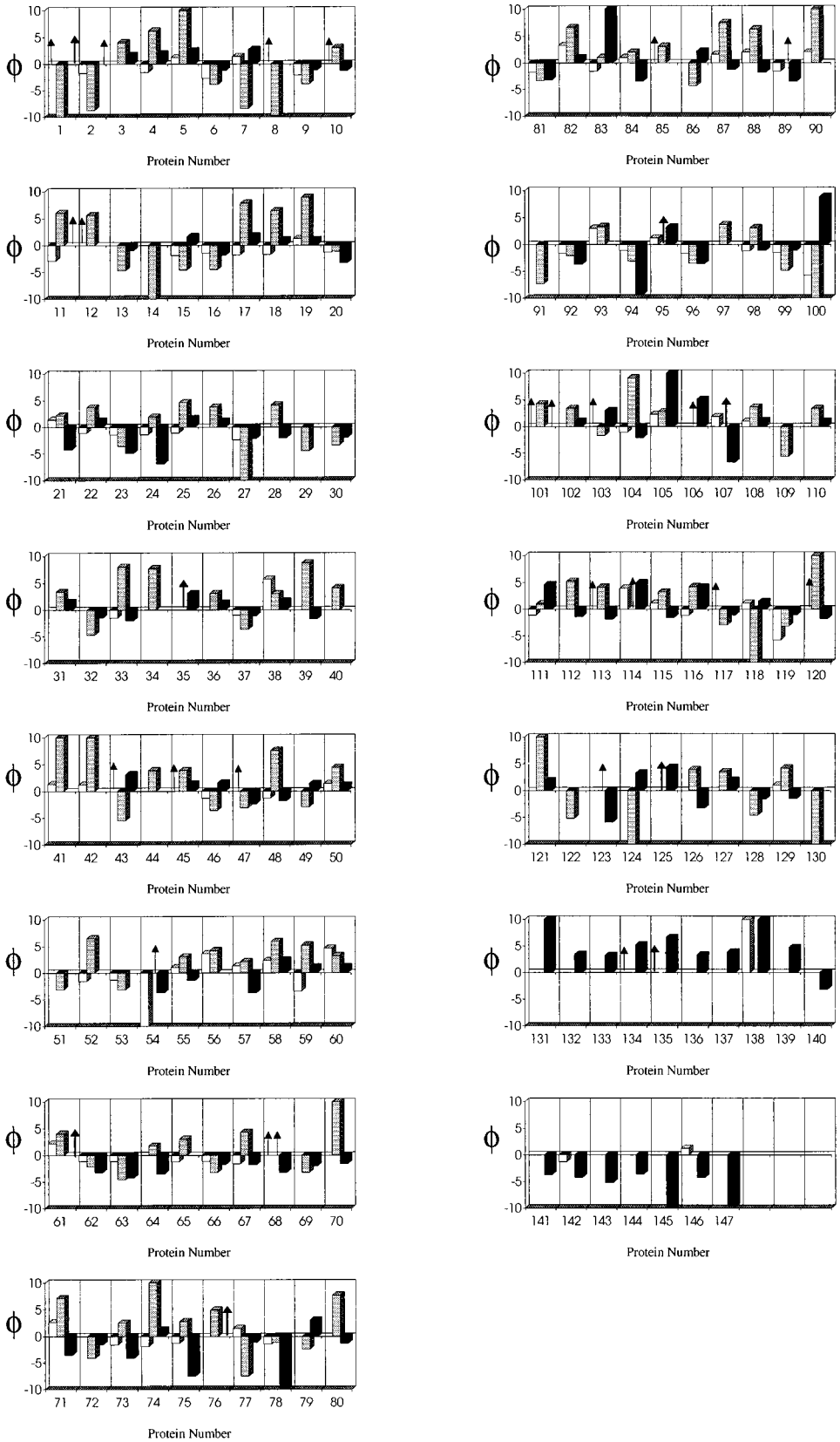

FIG. 3. Bar chart showing ratio of the relative concentration (ϕ) of individual proteins with isoelectric points in the range of pH 4 to 6 from cells of *P. putida* KT2442 exposed to 1.63 mM 2-chlorophenol to that in the corresponding untreated control culture at 45 (□), 65 (▨), and 95 (■) min after exposure. For simplification, values of ϕ greater than ±10 are shown as ±10. A positive value indicates that the protein was found at a higher relative concentration (i.e., the concentration of that protein expressed as a percentage of the total protein concentration measured on the scanned gel) in the 2-chlorophenol-treated cells than in the control (untreated) cells. An arrow is used to indicate that the protein was present in the 2-chlorophenol-treated cells but absent from the corresponding control (the length of the arrow is arbitrary). Most of the proteins included in this comparison are shown in Fig. 2 and the numbers refer to the protein numbers in Fig. 2. The remainder refer to proteins only found on gels from samples taken at 45 or 95 min (data not shown).

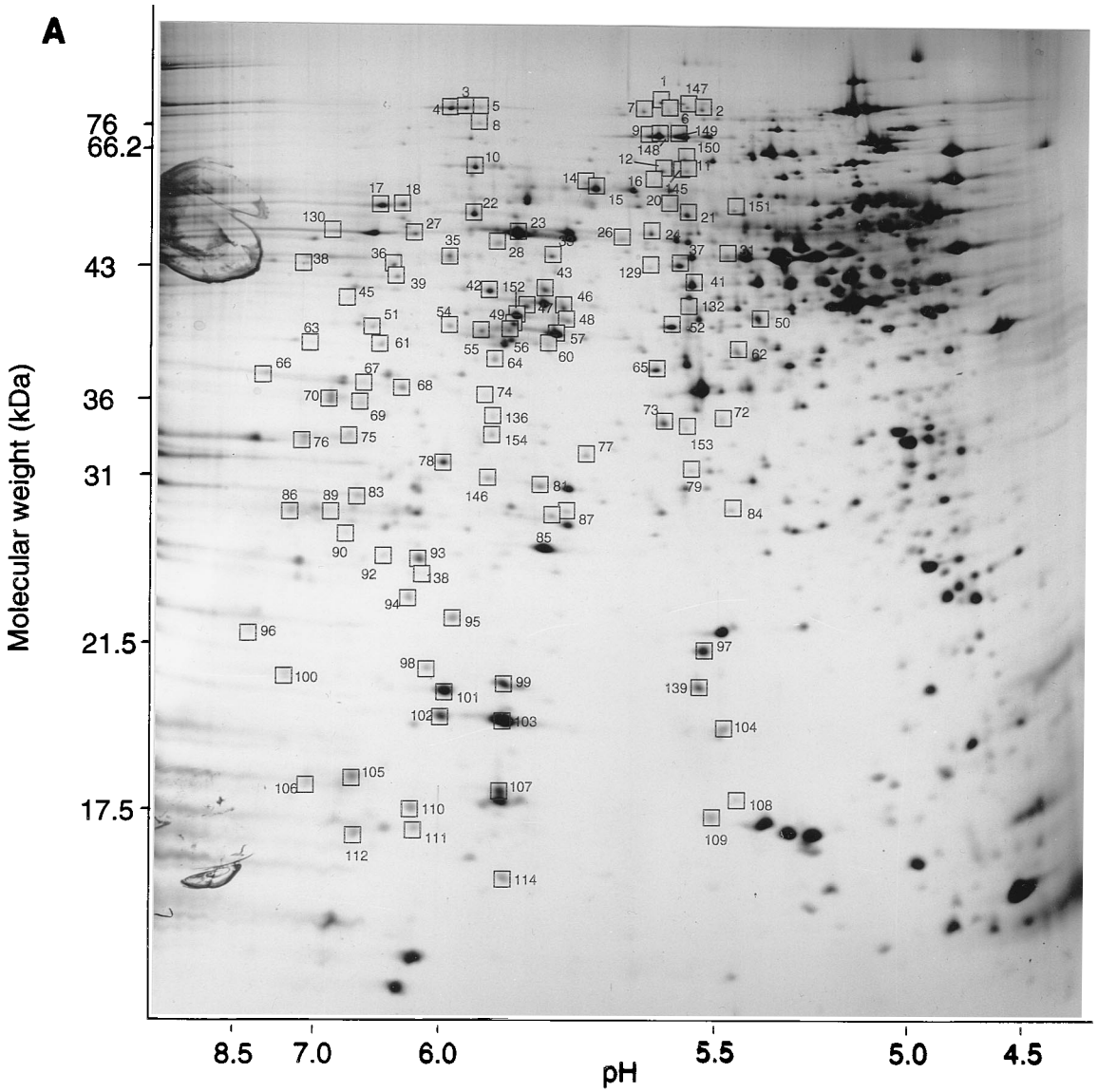

FIG. 4. Silver-stained 2D-PAGE of protein extracts of *P. putida* KT2442 from exponentially growing cells 95 min after exposure to 1.63 mM 2-chlorophenol (A) and from unexposed control cells in a parallel culture at the same point in time (B). All proteins are shown with isoelectric points in the range of pH 3.5 to 10. In panel A, those proteins with isoelectric points above ca. pH 5.5 which were present at concentrations of more than either a threefold excess or deficit compared with the corresponding protein concentrations in the untreated control at at least one of the sampling times (45, 65, or 95 min after exposure) are boxed and numbered and quantified in Fig. 5. Proteins with isoelectric points below pH 5.5 are examined in detail in the gels shown in Fig. 2. First-dimension isoelectric focusing was carried out by using linear Immobiline-fixed gradient gels. Second-dimension PAGE was carried out with linear gradient gels (11.6 to 17.4%).

found at one sampling time with a value of ϕ greater than 3 was found at a separate sampling time in the treated culture only and not in the parallel untreated control.

The following types of response are indicated by the protein levels in Fig. 3: (i) an increase in protein concentration with time compared with that in the control (no addition of 2-chlorophenol), e.g., protein no. 11 or 119; (ii) a decrease in protein concentration with time compared with that in the control, e.g., protein no. 8 or 60; (iii) a peak in protein concentration in either the control or the treated cells after which the concentration either increases or decreases, e.g., protein 27 or 74; and (iv) essentially unaltered protein concentrations during the experiment, e.g., all unmarked proteins in Fig. 2A.

Figure 3 indicates that of the 147 proteins whose concentrations differ by more than threefold from those in the control at some point in time following exposure to 1.63 mM 2-chlorophenol, 30 belong to class I, 27 belong to class II, and 90 belong to class III.

Comparison of proteins with isoelectric points above pH 5.5. Since the linear IPG strips allow separation of the proteins with isoelectric points between pH 4 and 6, they exclude all those proteins with higher isoelectric points. Use of the nonlinear IPG strips with a pH range, as described by the manufacturer, from 3.5 to 10 allows these proteins to be resolved. The 2D-PAGE patterns of the samples taken at 95 min after the addition of 2-chlorophenol as well as of the parallel control are shown in Fig. 4. For the gels with a broader pH range, the composition of the pH gradient in the IPG strips used in isoelectric focusing was such that there was a rapid increase in the pH along the length of the gel between ca. pH 3.5 and 5,

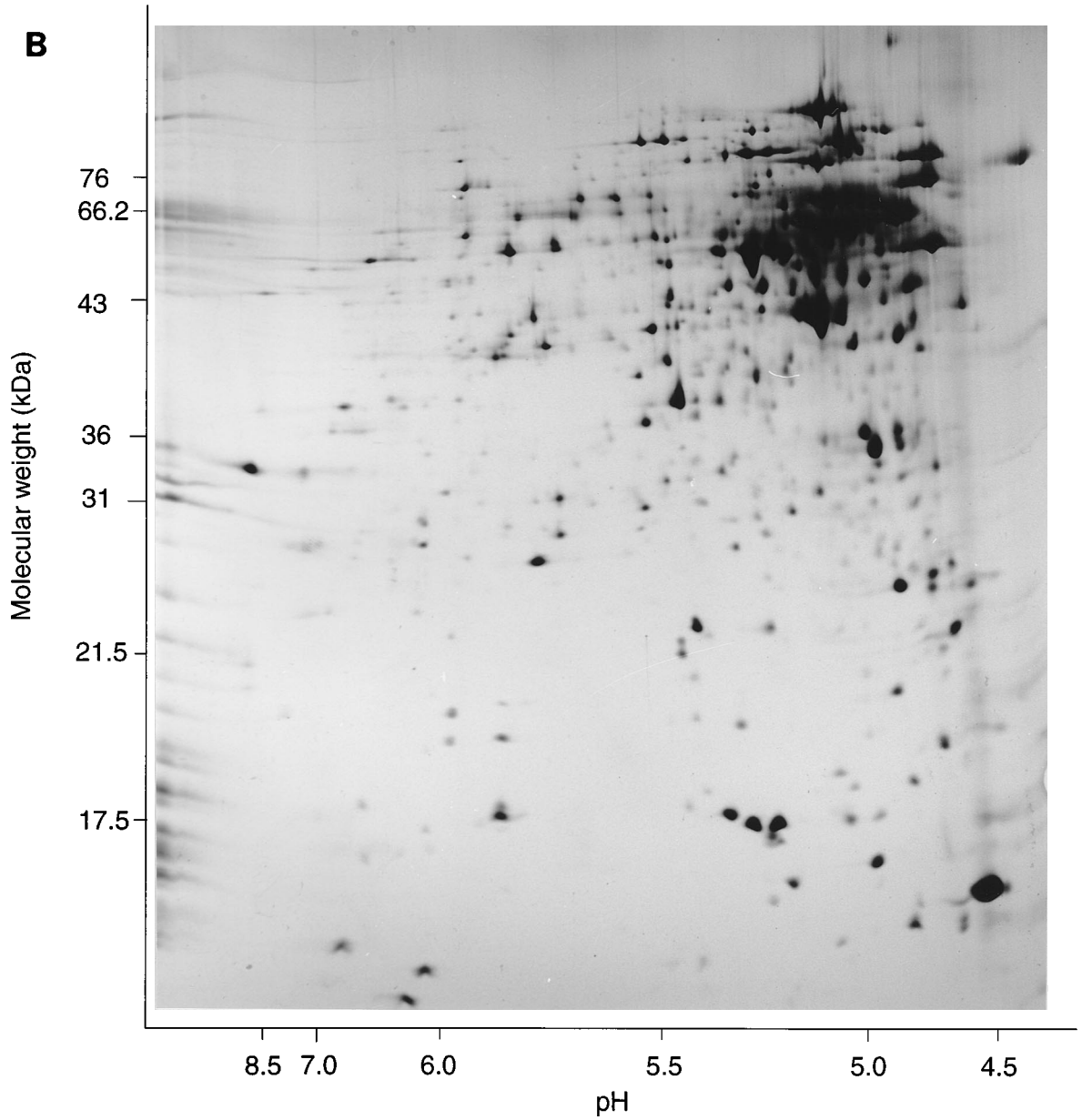

FIG. 4—*Continued.*

after which the pH increased more gradually to pH 6. The latter part of the pH range also had a steep gradient. This is reflected in the nature of the pH scale shown on the *x* axis of the gels. Since the resolution of the proteins in the range of pH 4 to 5.5 is inferior on these gels compared with those shown in Fig. 2, only proteins with an isoelectric point greater than ca. pH 5.5 were considered. There is, as a consequence, a limited amount of overlap between the gels obtained by using a narrow pH gradient in the first dimension and those shown here by using the broader-range pH gradient, as seen in Fig. 4.

Analogous to the method of comparisons described above for gels with a narrow pH range, Table 2 provides a summary of the major differences observed between the 2-chlorophenol-treated and untreated cells of *P. putida* KT2442. In Fig. 5, the concentrations of those proteins with concentration differences (ϕ) greater than threefold are shown for the samples taken after 45, 65, and 95 min. As in the classification system described above, 68, 39, and 47 proteins could be assigned to classes I, II, and III, respectively.

Reproducibility. On the gels covering the pH range from 4 to 7, there was an average of 712 spots per gel, and on the gels covering the range from pH 3.5 to 10, there was an average of 855 spots per gel. The range of proteins per gel however showed quite a lot of variation, i.e., for the linear pH gradient gels, a minimum of 589 and a maximum of 858; for the nonlinear broad pH gradient gels, a minimum of 655 and a maximum of 1,121. These differences in resolution are inevitable as a consequence of minor variations in the running and staining conditions. However, the effect that these differences have on the results can be estimated to be relatively slight. This was shown by running parallel gels of the same sample and pairing the gels. In one gel, 86% of the proteins could be paired, and

TABLE 2. Number of proteins with isoelectric points between pH 5.5 and 10 whose relative concentrations changed as a result of addition of 2-chlorophenol

Sampling time (min)[a]	No. of proteins with increased concn in[b]:		No. of proteins found only in[c]:	
	Treated cells	Untreated cells	Treated cells	Untreated cells
45	37	20	6	7
65	29	17	1	2
95	67	5	4	2

[a–c] Footnotes as in Table 1.

in the second gel, 79% could be paired. Of those proteins paired, only 3 and 1%, respectively, showed a significant ($\phi >$ 3) difference in concentration. The proteins which could not be matched on the gels represented only 4 and 6%, respectively, of the total protein present on the gels.

Determination of free 2-chlorophenol concentration during stress experiments. In order to be certain that the cells of *P. putida* KT2442 were exposed to the same concentration of 2-chlorophenol during the entire experimental period, and to determine whether any degradation or other physical loss of 2-chlorophenol occurred, the concentration of 2-chlorophenol was measured at various times following its addition to an exponentially growing culture of *P. putida* KT2442. The results (data not shown) indicated that the 2-chlorophenol concentration remained constant and no biodegradation took place.

DISCUSSION

In most cases in which stress protein expression has been monitored, 2D-PAGE has been used as the method of choice with autoradiographic detection of protein synthesis following incubation of samples with labelled amino acids. As such, the stress proteins themselves have not necessarily been directly addressed since enhanced synthesis does not necessarily reflect enhanced concentration in the cell. Thus, if the degradation rates of proteins are enhanced, the cellular level can be maintained only if the synthesis rate is also enhanced. Since, especially in the case of heat shock, some proteins whose synthesis rates are enhanced are proteolytic (17), and errors in protein synthesis occur more frequently under stressed conditions (7), it is likely that higher synthesis rates of certain proteins are necessary if they are vital to the survival of the cell. This essentially ensures not an enhanced protein level but a stable protein level. As such, it is of considerable interest to be able to look at the protein concentrations per se following a stress event. This has been possible for the case of individual proteins whose function as a stress protein has been shown but not for the wider global response network. One reason for this limitation was the poor resolution of proteins by using the classical O'Farell 2D-PAGE method (24) and standard silver staining. The various improvements in the techniques of 2D-PAGE (2, 9, 24) and in the silver staining protocols (2) have now made this possible, and the results of one such analysis are presented here.

The analysis of the proteins presented in the tables and figures in this article suggest that four different categories of proteins are present. These categories are based solely on the trends in the comparative concentrations of the proteins in 2-chlorophenol-treated cultures and their parallel controls. Proteins of classes I and II include two subsets of different protein responses. One is made up of those proteins which were either suppressed or activated early after exposure to 2-chlorophenol but whose concentrations approached the concentrations found in the control after extended exposure. The more extreme response involves those which show a longer-term response, i.e., those which increase or decrease to significantly different levels compared with corresponding protein concentrations in the control by the end of the sampling period. The transient response pattern has been also seen in many other stress-type responses. For example, in the heat shock response the level of synthesis of the heat shock proteins rapidly increases within 10 min of the change in temperature, and then synthesis slows (19). After an extended period of exposure to enhanced temperatures, the concentration of each individual heat shock protein stabilizes at a new level indicative of the temperature of exposure (12, 13). In the case of starvation stress, there is a cascade of proteins which appear to be activated and deactivated depending on the duration of the starvation condition (16). Thus, a pattern of protein rearrangement as seen here with the chemical stress response is not unexpected and probably reflects the functions of the proteins involved and the need for an initial rapid adaptation to protect the cell against the damaging influence of the stress followed by a period of functional metabolic enhancement during which the proteins expressed modulate metabolism under the altered physical or chemical conditions imposed.

In the proteins described here as belonging to either class I, II or III, only four of the proteins with isoelectric points between 4 and 7 are consistently different (protein no. 23, 63, 94, and 105), while of those proteins with higher isoelectric points, 12 fall into this category (protein no. 3, 5, 8, 45, 63, 74, 89, 96, 98, 100, 106, and 110). In addition, there are many more proteins for which enhanced or lower concentrations were found in the two latter sampling points while after 45 min the concentrations differed little from those of the controls. This latter effect reflects the type of response described by Nyström et al. (22) for the starvation survival response in *Vibrio* sp. strain S14.

At present, little is known about the nature of the 2D protein patterns for *P. putida* KT2442, and very few of the proteins have been identified. Only two such studies have been published (8, 15), and identification of these proteins must follow in the near future. This is in contrast to *E. coli*, for which a limited number of the proteins on 2D gels have been identified (29).

The chemical stressing agent used here, 2-chlorophenol, is effective as an uncoupling agent. Its effect can therefore be expected to be directly on the membrane, and it is unlikely to be intracellularly active, although it is possible that 2-chlorophenol can bind to proteins if it enters the cell. The growth rate reduction observed following treatment with 2-chlorophenol is likely to have occurred as a result of the dissipation of the membrane potential. The importance of the changes in the protein concentrations observed will eventually have to be related to the mode of action of the uncoupling agent and the secondary and tertiary effects of this action. With the stronger uncoupling agent 2,4-dinitrophenol, significant changes in the protein expression pattern have also been demonstrated (6).

This work has focused on the chemical 2-chlorophenol. It serves here only as a model to provide preliminary data as to the type of response which occurs. However, this compound is of environmental relevance since it has been estimated that of the 20,000 tons produced synthetically each year, some 2,000 tons end up in the environment (26). In addition, it is a by-product of microbial transformation reactions of chlorobenzenes and arises during the chlorination of drinking water and wastewater. In the future, it will be necessary to look at the reactions of a wide range of other chemicals in order to obtain

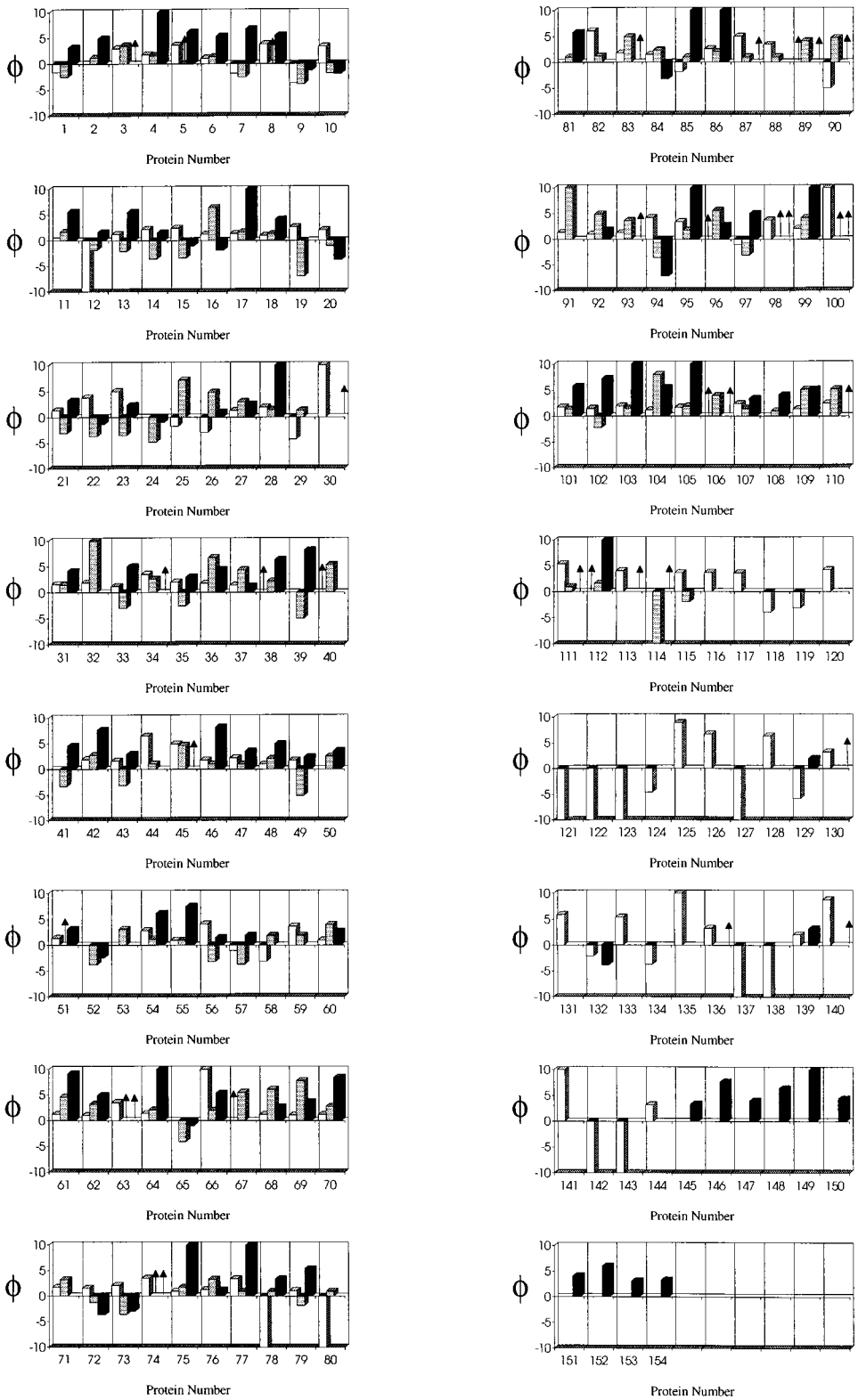

FIG. 5. Bar chart showing ratio of the relative concentration (ϕ) of individual proteins with isoelectric points in the range of pH 5.5 to 10 from cells of *P. putida* KT2442 exposed to 1.63 mM 2-chlorophenol compared with that in the corresponding untreated control culture at 45 (□), 65 (▨), and 95 (■) min after exposure. For simplification, values of ϕ greater than ±10 are shown as ±10. A positive value indicates that the protein was found at a higher relative concentration (i.e., the concentration of that protein expressed as a percentage of the total protein concentration measured on the scanned gel) in the 2-chlorophenol-treated cells than in the control (untreated) cells. An arrow is used to indicate that the protein was present in the 2-chlorophenol-treated cells but absent from the corresponding control (the length of the arrow is arbitrary). Most of the proteins included in this comparison are shown in Fig. 4, and the numbers refer to the protein numbers in Fig. 4. The remainder refer to proteins found only on the gels from samples taken at 45 or 65 min (data not shown).

a clearer understanding of the chemical stress response in general. In *E. coli* there are already proteins which appear to be important under a wide range of stress conditions (20–22), including exposure to 2,4-dinitrophenol (6), and similar universal stress proteins are already known to exist in other bacteria (30).

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