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A Comparative 2-Dimensional Gel Protein Database of the Intact and Regenerating Newt Limbs

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A comparative two-dimensional gel protein database of the intact and regenerating newt limbs

In this paper we describe a two-dimensional gel database of the regenerating newt limb. Protein synthesis was compared in the intact limb, in the 1-week regenerating limb, representing the dedifferentiation stage, and in the 2-week regenerating limb, representing the formation of the blastema. This comparative database provided data on differential expression of about 800 proteins during the process of limb regeneration. In addition, a map has been generated for these proteins for future guidance in characterizing further new, unknown proteins. The overall expression patterns of the proteins indicated that the dedifferentiation stage was marked by down-regulation of most proteins, while the blastema formation was marked by the appearance of many new proteins. The potential use of such a database in isolating factors involved during limb regeneration is discussed.

1 Introduction

Developmental processes are usually governed by regulatory events at the gene level. Such regulation is responsible for gene transcription and subsequent protein synthesis. One method of understanding and characterizing the molecules that play significant roles during developmental phenomena is the generation of protein databases whereby the presence or absence of particular proteins can be assessed as a consequence of a certain developmental phenomenon. Amphibian limb regeneration during adulthood is a unique developmental event confined only to some urodeles. The process is initiated by the covering of the amputated limb by epithelial cells, the wound epithelium. It is histologically apparent that the remaining terminally differentiated tissues dedifferentiate and form a cell population, called the blastema, which shows characteristics of embryonic cells. This remarkable return of adult to embryonic cells is of enormous biological importance in understanding cell differentiation and cancer. The blastema cells, after a period of proliferation, redifferentiate to the tissues (such as cartilage and muscle) and reconstitute the missing part [1]. The role of stem cells specific for giving rise to blastema has not been documented, so far, but it should not be ruled out. The identification of key molecules that portray the different stages of this phenomenon is of utmost importance. Hence, we initiated studies to map protein synthesis in the intact, dedifferentiated and regenerating limb of the newt in order to create a database that would guide us in comparing expression of proteins and eventually characterize them. Knowledge of protein synthesis in the regenerating newt limb, or in the newt in general, is poor. The goal of the present study was to compare protein synthesis during the regeneration process. This approach, in conjunction with other molecular and cell biology methods, could provide the means for isolating and defining those factors that play a role in limb regeneration.

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Abbreviations: 2-D, two-dimensional; **DPM,** disintegrations per minute

2 Materials and methods

2.1 Animals

Adult newts (*Notophthalmus viridescens*) were purchased by Amphibian of North America (C. Sullivan, TN, USA). Forelimbs were amputated bilaterally at the level of the elbow. The removed parts were the intact limb samples.

2.2 Protein isolation and two-dimensional polyacrylamide gel electrophoresis

[^{35}S] Methionine (1 mci) was injected intraperitoneally 2 days before collection of the tissues. The tissues collected were intact limbs, 1-week blastema and 2-week blastema, including the wound epithelium. The isolated tissues were initially rinsed several times with cold buffered saline, pH 7.5. Tissues were crushed in liquid nitrogen and solubilized by adding a hot SDSBME solution, 0.1% containing sodium dodecyl sulfate (SDS), 20 mM Tris, pH 8.0, 5% 2-mercaptoethanol (Protein Databases Inc., Huntington Station, NY, USA). The tubes containing the samples were then cooled and 0.1 volume of a solution containing DNAase (1 mg/mL) and RNAase (500 μg/mL) was added, and the tubes were incubated for 2 min on ice. The samples were then frozen in liquid nitrogen, ready to be analyzed by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). Gel electrophoresis was carried out by the method of Garrels [2]. For isoelectric focusing, 2% carrier ampholytes (pH 4–8) were used (BDH Chemicals, Poole, Dorset, UK). The second-dimensional slab gels were 12.5% acrylamide gels. At the end of electrophoresis, the gels were fixed in 25% methanol/15% acetic acid for 1 h and processed for autoradiography at −70°C. Quantification and statistical analyses of the protein patterns were performed using the PDQUEST software (Protein Databases Inc.). This software can provide information on the quantification of each protein at different stages. According to the procedure, spot quantification and resolution of overlapping spots is performed by 2-D Gaussian fitting. The matching of the patterns revealed by the autoradiograms is carried out for groups of gels, called matchsets, and within each matchset every gel is matched to every other gel. Tests have shown that up to 97% of the spots in each pattern can be matched and that fewer than 1% of the spots are matched inconsistently. The reproducibility of all aspects of this 2-D gel analysis by using the QUEST program has been treated by Garrels [2, 3].

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3 Results

3.1 Protein synthesis in the intact and regenerating newt limbs

Approximately 800 proteins were resolved by the 2-D gel analysis of the intact, 1-week and 2-week blastema. Figure 1 shows a computer-generated map which presents all the proteins from the three different stages of limb regeneration. From these data we can deduce the specific protein synthesis during each stage. Related to this, we found that 117 proteins are synthesized exclusively in the intact limb, 26 exclusively in the 1-week blastema, while 134 are distinct to the 2-week blastema. Synthesis of other proteins was found not to be unique to one stage but common to any two or all three stages: 15 proteins were found to be common to the intact limb and 1-week blastema, 202 proteins common to the intact limb and 2-week blastema, 53 proteins common to 1-week and 2-week blastema, and 243 proteins common to all three stages. While the blastema formation (2-week sample) is characterized by enormous synthesis of new proteins, the 1-week blastema (dedifferentiation stage) is marked by a significant reduction in new protein synthesis. It is apparent from these results that the disappearance of proteins is fundamental for the dedifferentiation stage. Figure 2 shows another computer map printout with a disintegrations per minute (DPM) cutoff for easier orientation in the map, and Fig. 3 shows an autoradiogram from a 2-week blastema sample. Note that some of the new proteins appearing during blastema formation could be modified forms of the already existing proteins (*i.e.* phosphorylated). In that case these modifications could be of importance for the function of the proteins and will therefore need to be elucidated.

3.2 Comparison of protein expression during the different stages

The proteins from the three different stages were quantified using the QUEST program (see Section 2.2). Once the database had been generated, each spot was compared in the three different stages so that the pattern of expression of each protein became known. Quantification was performed in duplicate in order to receive a more accurate picture. The expression of each spot was plotted as a graph. An example of such a graph is given in Fig. 4. The expression (DPM counts) is depicted as lines. The spot number and the counts are shown at the lower left of the graph and at the right top, respectively. This analysis facilitates visualization of the expression of each protein as well as direct comparison during the three different stages. In Appendix 2, we present this analysis for all proteins of the database shown in Fig. 1.

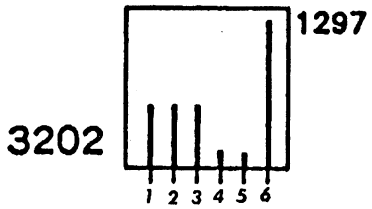

Figure 4. A sample graph showing the expression of one protein during limb regeneration. This protein is a keratin homologous to Xenopus type I keratin B2 [4, 5]. Lines 1 and 2 indicate the expression from two different experiments in 1-week blastema, lines 3 and 6 in 2-week blastema and lines 4 and 5 in the intact limb.

4 Discussion

This is the first detailed analysis of the comparative protein database of intact and regenerating newt limbs. This analysis has provided unique information about protein synthesis and regulation during the process of dedifferentiation and blastema formation. The fact that dedifferentiation is marked by disappearance of proteins could indicate an important biological event in regeneration, namely that the down-regulation of protein synthesis could be a crucial event for the regeneration to take place. Having mapped the proteins we can now analyze them by microsequencing. Protein 3202, for example, was fount to be a keratin [4, 5], and protein 1111 is beta actin [6]. Such sequence information could allow us to create probes and isolate the genes. The database will lead us to isolation and characterization of proteins with interesting patterns of expression during the regeneration process. Other databases (*i.e.* from the nervous system, known to influence limb regeneration) could also be useful in elucidating cellular and molecular mechanisms [7, 8]. Such work has now begun in our laboratory and it is hoped that crucial factors involved in amphibian limb regeneration will be isolated.

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5 References

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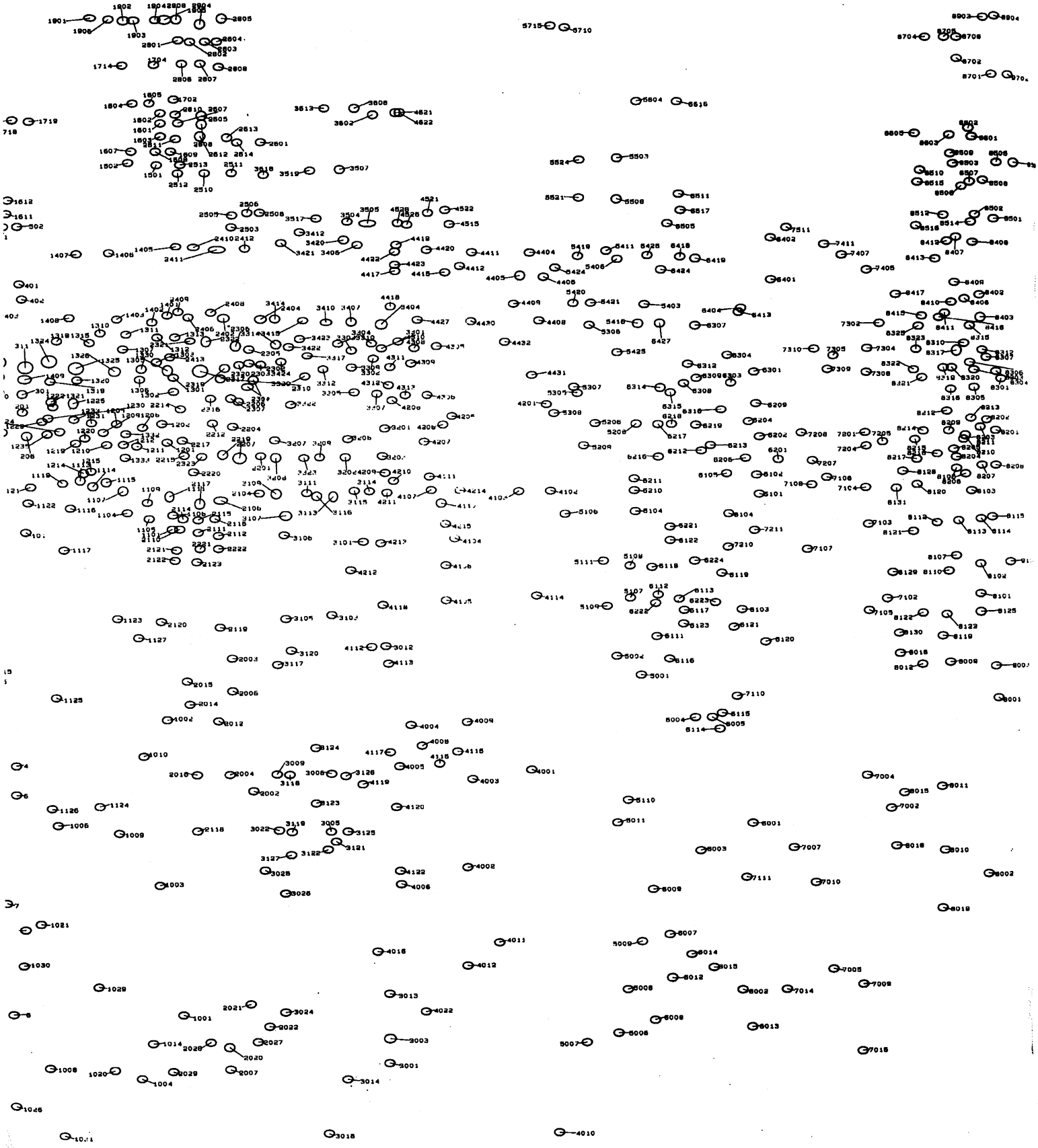
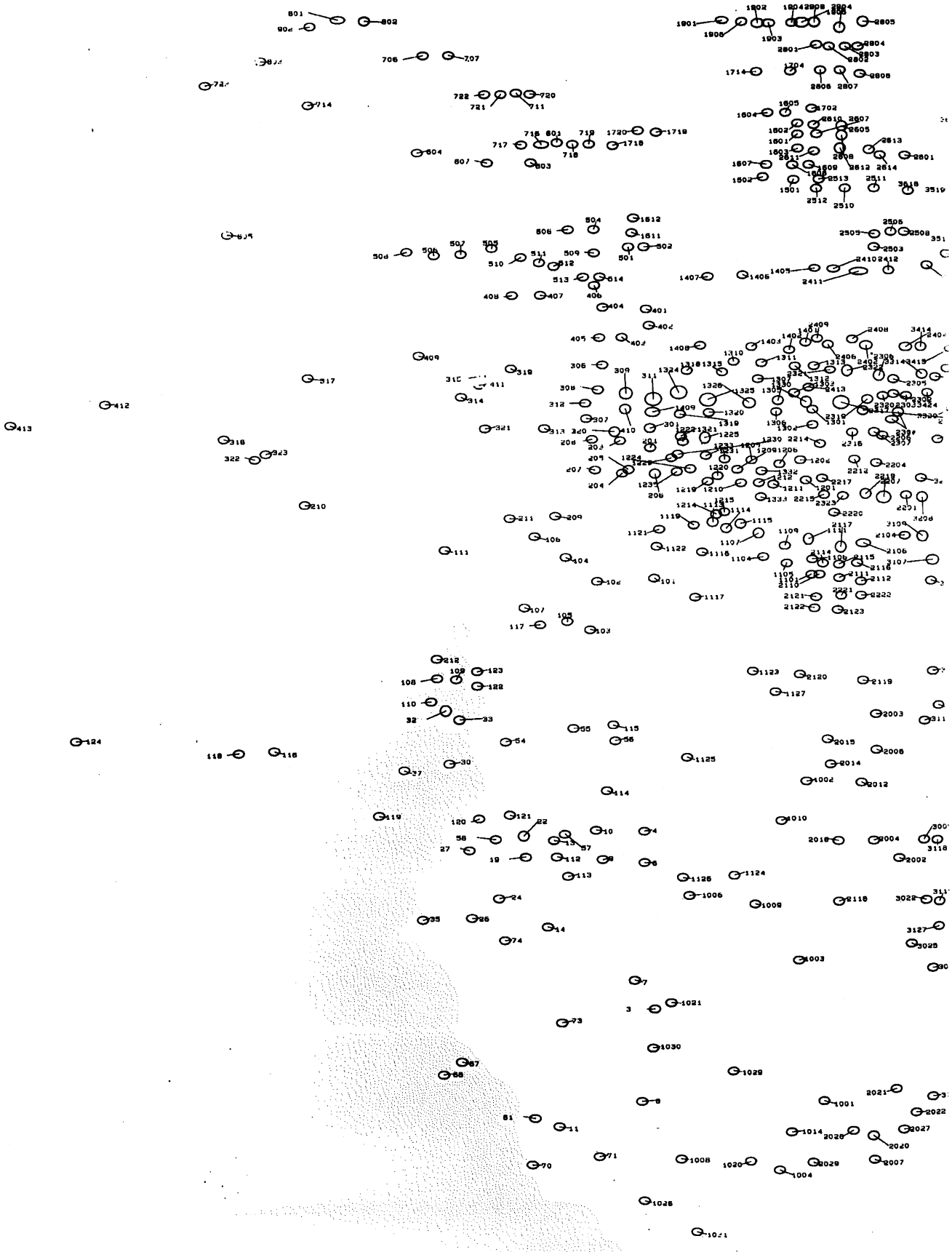
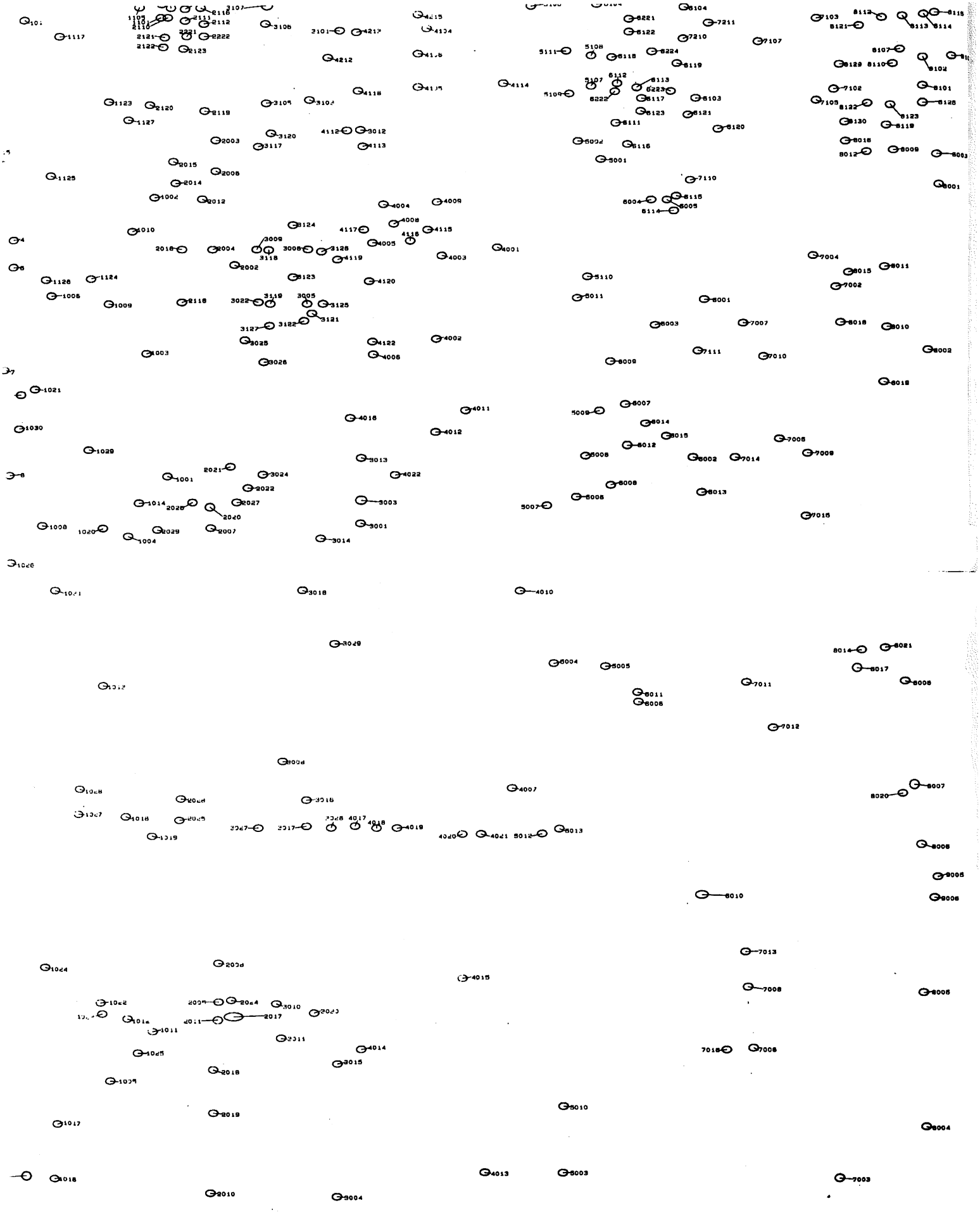
Electrophoresis 1993, *14*, 148–156

6 Appendix 1: The 2-D gel database from the intact and regenerating limb (Figs. 1–3)



6 Appendix 1: The 2





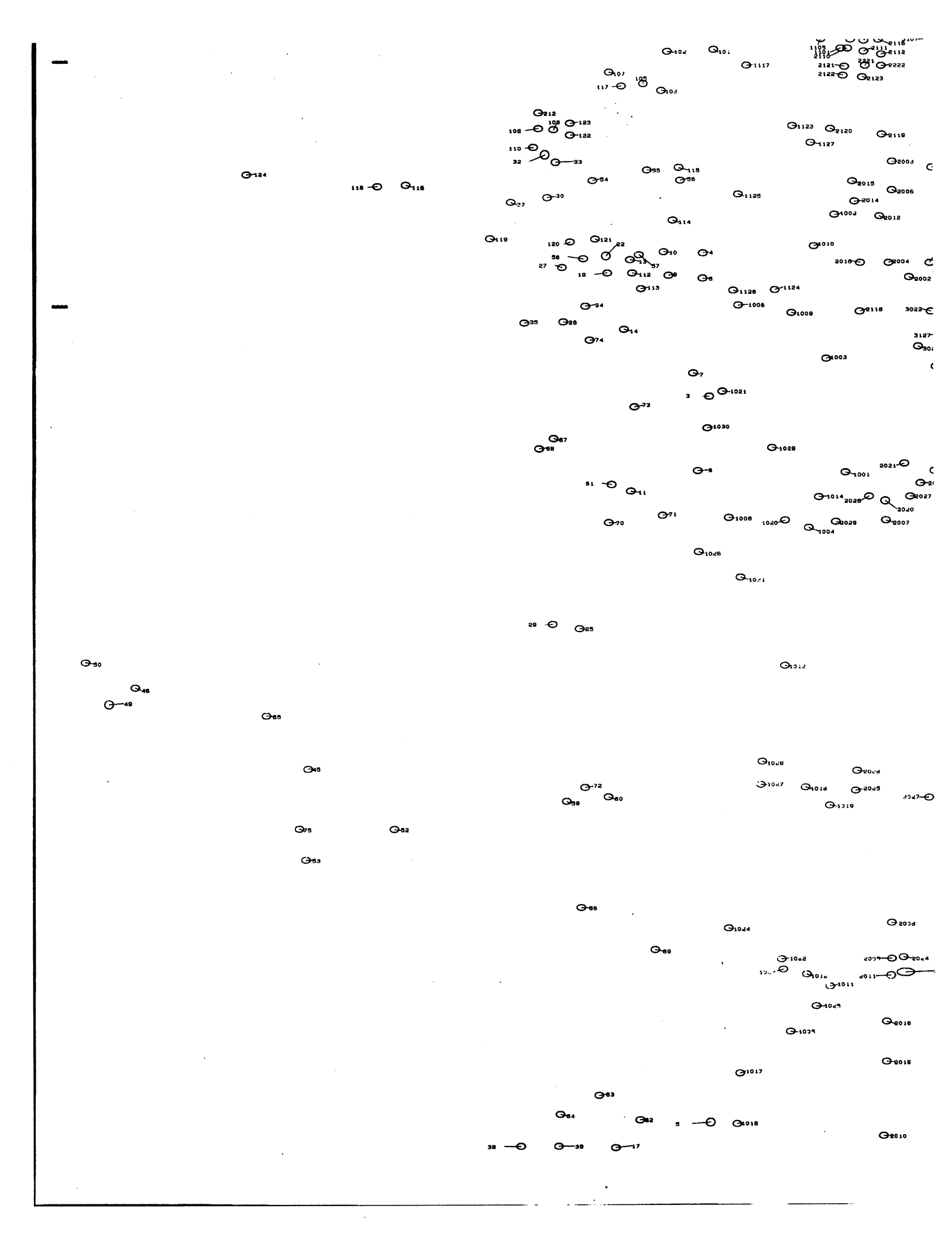

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Figure 1. Computer printout of all poypeptides resolved in the 2-D gel analysis from the intact, 1-week and 2-week regenerating newt limb. The bars indicate molecular masses 30, 45, 60 and 200 kDa.

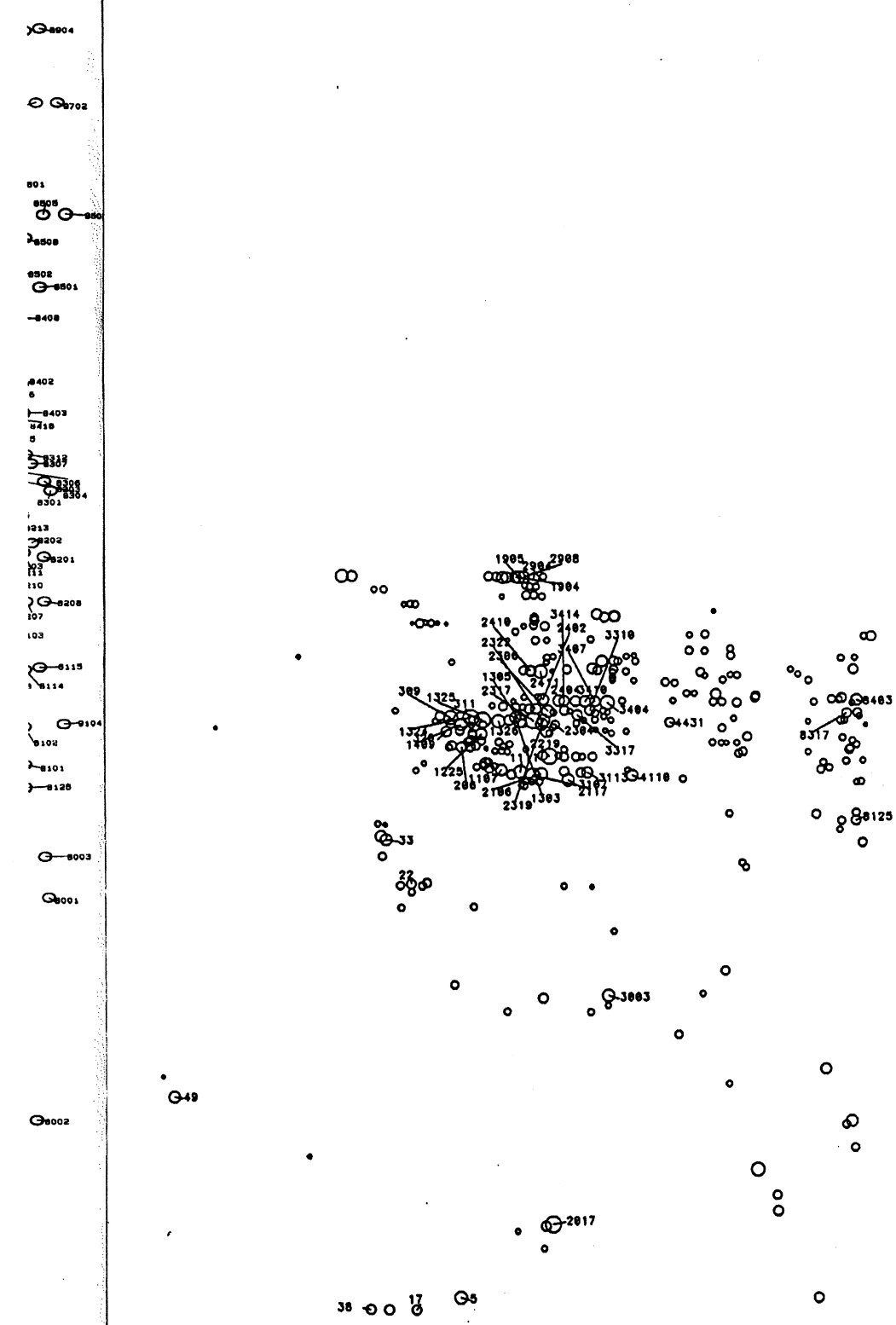


Figure 2. Computer printout with a DPM cutoff of 37 of the polypeptides shown in Fig. 1; 300 polypeptides are presented.

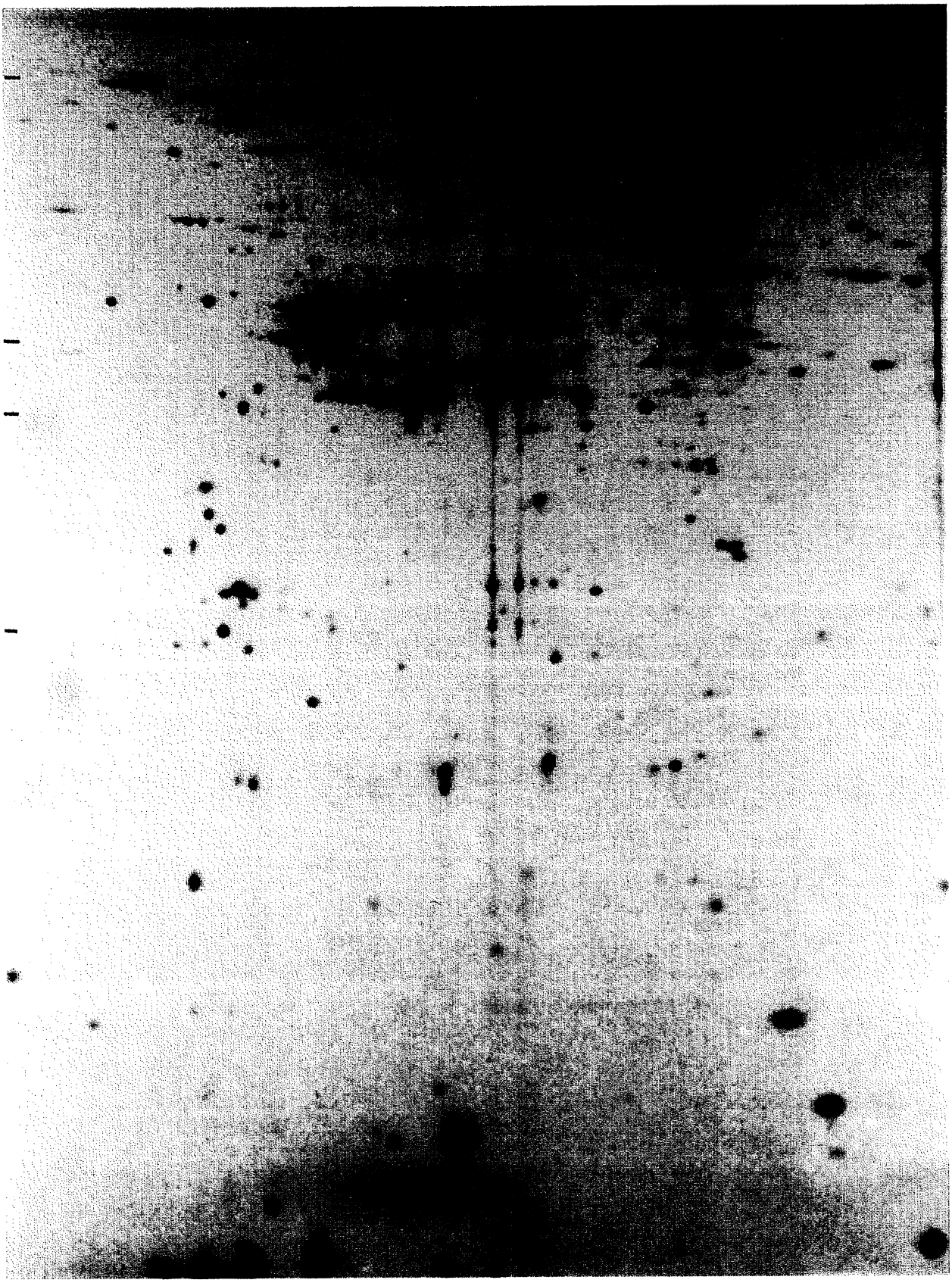
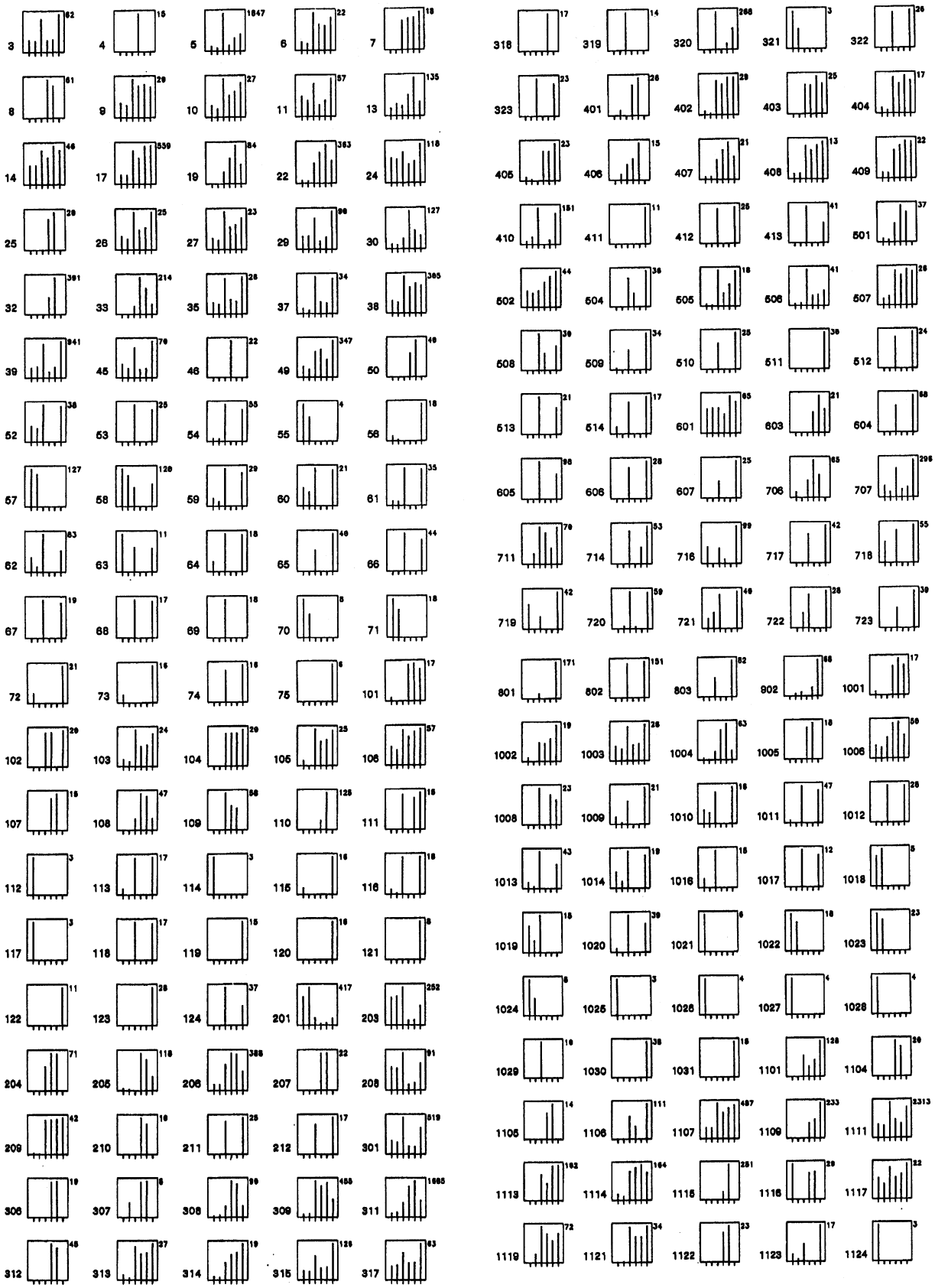
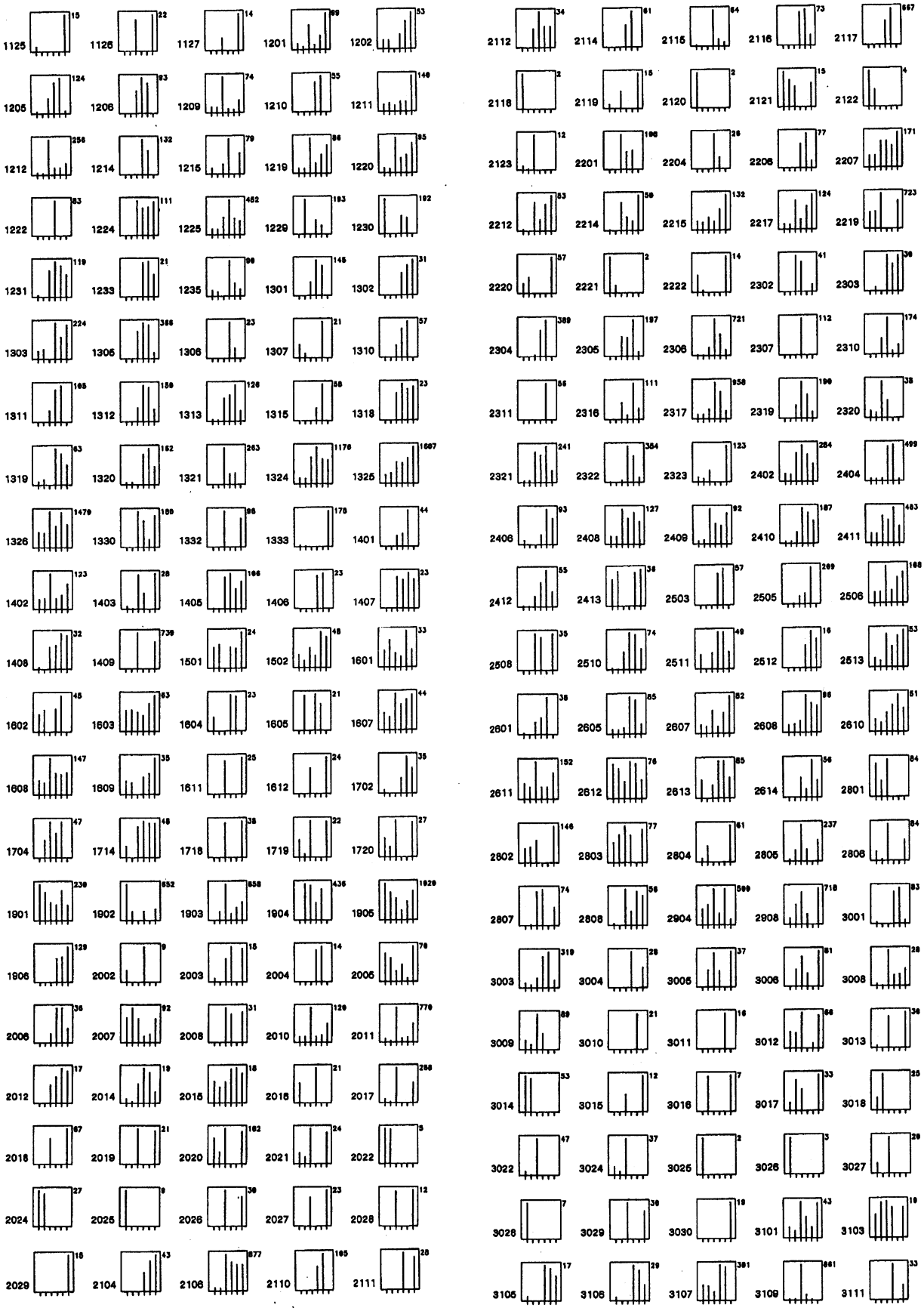


Figure 3. 2-D gel autoradiogram of [^{35}S] methionine-labeled polypeptides from 2-week blastema. The pH is linear from 4 (on the left) to 6.8 (on the right), and is the same in Fig. 1 and 2. The bars indicated molecular masses as in Fig. 1.

7 Appendix 2: Expression of the polypeptides shown in Fig. 1 in the intact limb, as well as 1-week and 2-week regenerating new limbs

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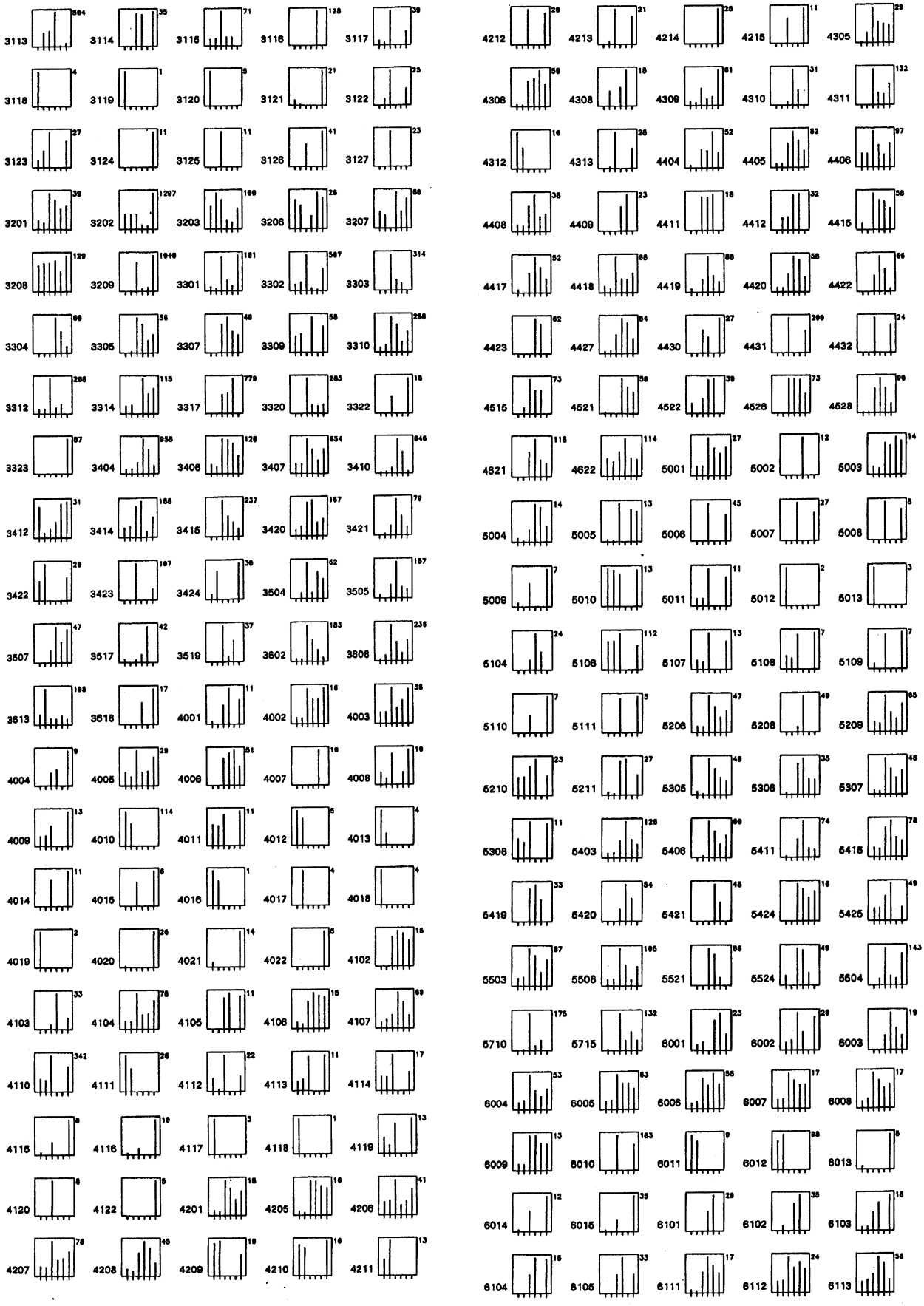
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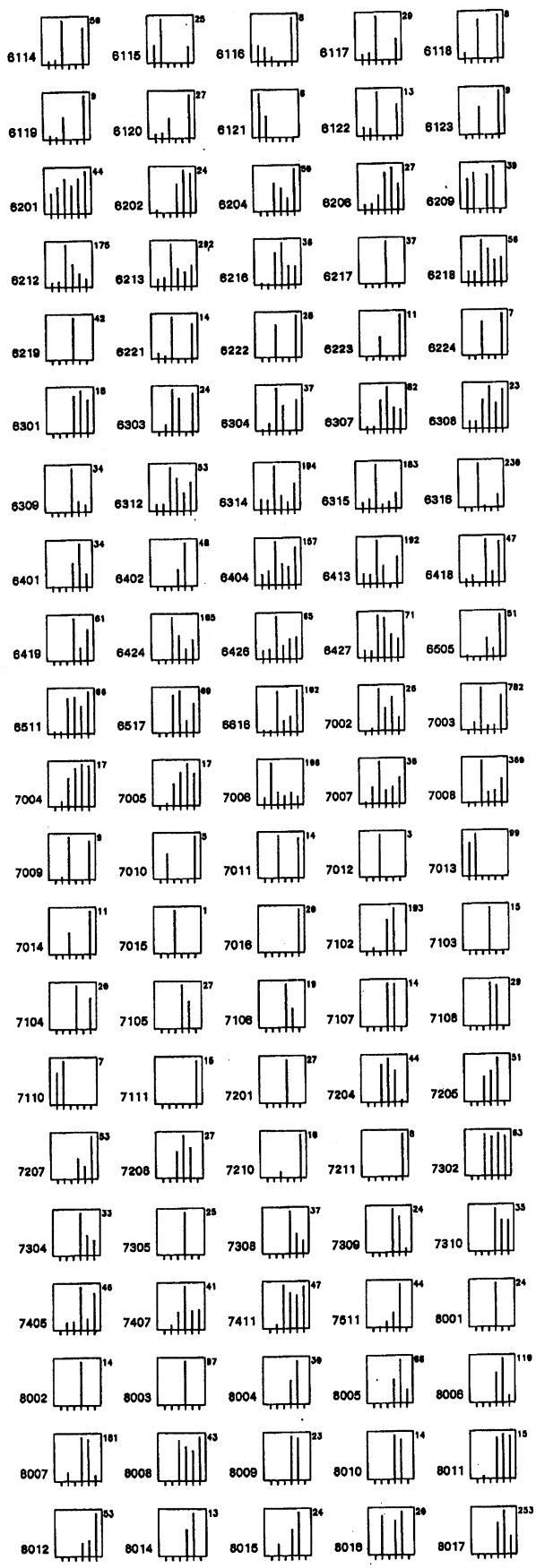
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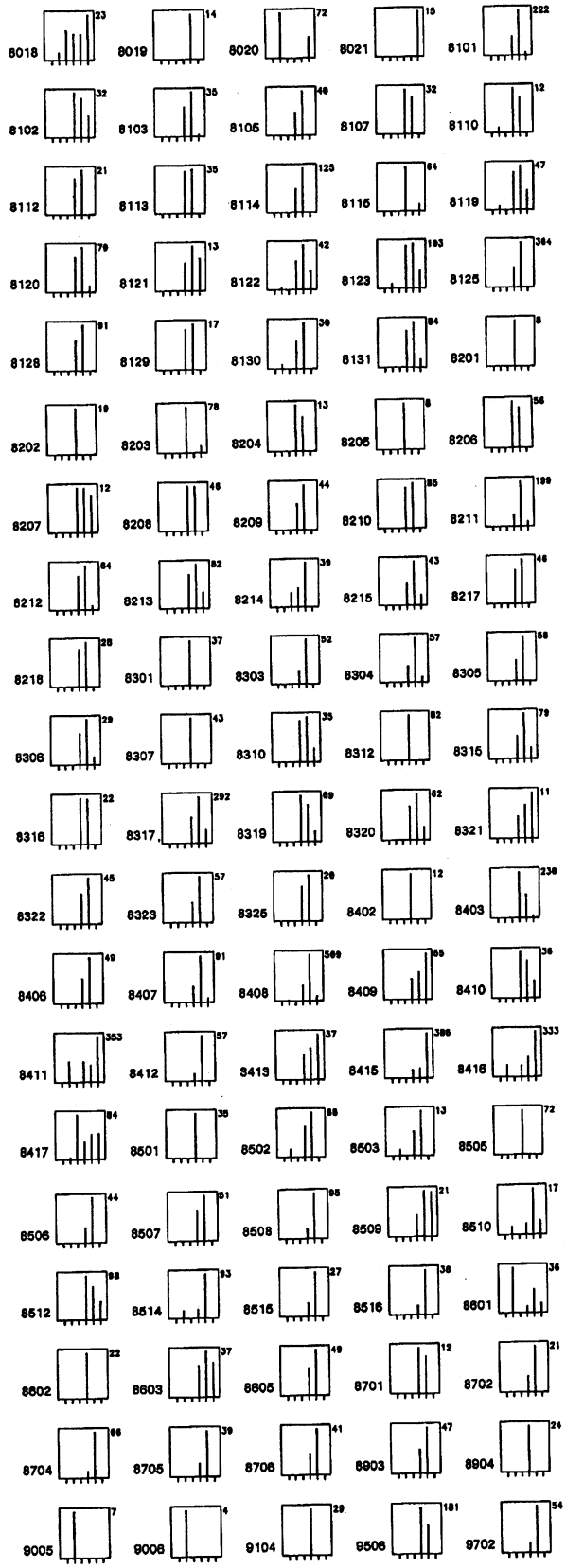
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