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Proteins of the brain and body wall in larvae of *Drosophila melanogaster*

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Abstract. Proteins of the brain and body wall cells of third instar larvae of *Drosophila melanogaster* have been examined by two-dimensional gel electrophoresis. Out of over 600 [^{35}S]-labelled peptide spots seen in brain or body wall extracts, 517 were common to both; 61 spots were unique to brain and 66 unique to muscle. Glycoproteins were identified by soaking the gels in radioactive iodinated Concanavalin-A. Forty four Con-A binding glycoproteins were identifiable in the brain and 41 in the muscle extracts. Out of these, 8 glycoproteins of the brain and 8 of muscles appear to be tissue-specific.

Keywords. Proteins; glycoproteins of brain; muscle; *Drosophila* larvae; 2-dimensional gel electrophoresis.

Introduction

Since the isolation by Benzer (1967) of non-phototactic mutants.a variety of behavioural mutants of *Drosophila melanogaster* have been found (Reviews by Benzer, 1971, 1973; Pak and Pinto, 1976; Ward, 1977). Identification of biochemical lesions in neurological mutants is expected to throw light on molecular mechanisms underlying behaviour. Of particular interest, from this point of view, are the proteins of the nervous system. Two-dimensional gel electrophoresis provides a convenient way of looking for altered proteins in mutants (O'Farrell, 1975). Our group is interested in temperature-sensitive paralysed mutants of *Drosophila* some of which appear to be affected in nerves or muscles (Siddiqi and Benzer, 1976; Ikeda *et al*., 1976; Wu *et al.*, (1978). As a ground work for comparing mutants with normal flies, we have examined the two dimensional (2D) maps of proteins and Con-A binding glycoproteins of the brain and body wall of wild type larvae.

Tissiers *et al* (1974) and Arking (1978) analyzed the proteins *of Drosophila* larvae by one-dimensional electrophoresis and Rodgers and Shearn (1977) have examined the proteins of imaginal discs by two-dimensional electrophoresis. Using short periods of labelling, Tissiers *et al.* (1974) and Arking (1978) found that the protein patterns changed markedly at different stages of development. As our primary purpose was to maximise the chances of detecting mutational alterations, we fed the

Abbreviations used: SDS; Sodium dodecyl sulphate, PMSF: Phenyl methyl sulphonyl fluoride, Np40: Non ionic detergent (P40), Con.A: Concanavalin A; 2 D, two-dimensional;DNase; deoxyribonuclease; RNase, ribonuclease.

larvae on radioactive sulphur for prolonged periods to ensure uniform labelling. In this paper we present an account of [^{35}S]-labelled proteins and Con-A binding glycoproteins from larval brain and body wall. The body wall preparation consists predominantly of larval muscles.

Materials and methods

Strain

The experiments were carried out with the wild type strain Canton Special (CS) of *Drosophila melanogaster.*

Materials

Chemicals and solutions

Chemicals: $Na_2{}^{35}SO_4$ and Na^{125} I were obtained from Bhabha Atomic Research Centre, Trombay. Ampholines were purchased from LK Broma, Sweden, ultrapure urea from Schwarz Mann, New York USA, Nonionic detergent (NP40) from Fluka ulm, W. Germany and β -mercaptoethanol from E. Merck, Darmstadt, W. Germany. Sigma Chemical Co., St. Louis, MO, USA, supplied Tris-base, deoxyribonuclease (DNAase), ribonuclease (RNAase), agarose, Concanavalin-A (lyophilized powder) and cytochorme-*c*. Sodium dodecyl sulphate (SDS) was obtained from Pierce, Illinois, USA; Acrylamide, N-N'-methyl bisacrylamide and N,N,N',N' tetramethyiethylene diamine, from Eastman Kodak, Rochester, New York, USA and glycine from Fischer Scientific Co., Philedelphia, USA. The rest of the chemicals were of analaR quality obtained from Sarabhai Merck, Baroda.

Solutions: The following working solutions were essentially as described by O' Farrell (1975). (1) Lysis buffer: – 9.5 M urea, 2% (w/v) nonidet P40, 2% ampholines, pH 3·5-10 and 5% β -mercaptoethanol (b) Sonication buffer: 0.01 M Tris-HCl pH 7·4, 5 mM $mgCl_2$, 50 ug/ml pancreatic RNAase (c) DNAase-solution: 1 mg/ml solution in 0.01 M Tris-HCl pH 7·4 and 1 mM $MgCl_2$. (d) Phenyl methyl sulphonyl fluoride (PMSF):– 40mMstockin 95% ethanol (3) sample overlay solution: – 9M urea, 1% ampholines pH 3·5 –10. (f) Acrylamide solution for the 1st dimension – 28·38% w/v acrylamide and 1·62% bisacrylamide (g) Acrylamide solution for the 2nd dimension (29.2% acrylamide and 0·8 bisacrylamide. *Drosophila* ringer (Ikeda and Kaplan, 1970): NaCl128mM, KCl4·7mM, $CaCl_2$ 1.8mM, $Na_2HPO_4$0.74mM, $KH_2PO_4$0·35 mM.

Radioactive labelling of larvae

Larval proteins were labelled uniformly with [^{35} S] by feeding the larvae with yeast grown on $^{35}SO_4$. The feeding medium (0.2 ml) containing 10% dextrose, 5% sucrose and 1.5% agar was dispensed in 5 cm r 0.7 cm glass tubes. Fifty microlitres of radioactive yeast (3 r 10_8 dpm) was added and the medium was melted to allow mixing with yeast. Fifty early second instar larvae (about 60 h of age) identified by size and anterior spiracles (Bodenstein, 1965), were placed in each tube and kept at 23-24°C in a moist chamber. After 88 h of feeding, the larvae developed into late 3rd instar. The incorporation of [^{35} S] under these conditions was about 6 r 10_6 dpm per larva. Increasing radioactivity beyond 0.23 m C*i*/100 μl of medium causes visible damage to larvae. They remain small and stunted and die within 2 days.

Preparation of sample for electrophoresis

The labelled larvae were washed in *Drosophila* ringer and fed on cold feeding medium containing unlabelled yeast for 2 h in order to chase the undigested radioactive yeast. Each larva was again washed and transferred to a drop of ringer on a cavity slide kept on ice. The larvae were dissected under a microscope. The brains and body walls were cleansed of all extraneous tissues and transferred to separate 4 cm × 0.6 cm glass microhomogenizers.

Body walls of 10 larvae were homogenized in 60 μ 1 homogenising buffer (sonication buffer 25 μ1, DNAase 25 μ1, PMSF10 μ1). 48 mg of urea and 90 μl of lysis buffer were then added to make the final urea concentration 9M. About 50 brains were homogenized in 45 μ 1 homogenizing buffer (sonication buffer, 20 μ 1; DNAase 20 μ 1, PMSF 5 μ 1) followed by addition of 38 mg urea and 75 μ1 of lysis buffer.

The usual incorporation of [^{35}S] in the tissues was about 6 r 10$_5$ dpm/body wall and about 6 × 10^4 dpm/brain. The extracts were either loaded on the gel immediately or stored at – 40°C for subsequent use. These extracts could be stored upto a week without appreciable deterioration.

Electrophoresis

Two dimensional Polyacrylamide gel electrophoresis was carried out essentially as described by O'Farrell (1975) with certain modifications which are noted below: –

A 11·5 cm isofocussing gel containing Ampholines in the pH range 3·5-10 in 4% acrylamide was made in a 13 r 0·25 cm (internal diameter) glass tube. The gel was overlaid with lysis buffer and pre-run at 200 V for 20 min followed by 300 V for 25 min and 400 V for 35 min. The upper cathode compartment of the electrophoresis apparatus contained 0.02 M degassed NaOH and the lower compartment contained 0·0l M H_3PO_4.

About 30 μl of body wall extract or 70 μ1 of brain extract containing 8 × 10^5 dpm and about 25 μg protein, was loaded on the gel and overlaid with 20 μ1 of sample overlay solution. The proteins were electrofocussed at 400 volts for 16 h followed by 800 V for 1 h at room temperature (23°C).

The cylindrical gel was equilibrated in sodium dodecyl sulphate-buffer on a reciprocal shaker for 90 min at room temperature and laid on a 14 r 11 r 2·25 cm SDS slab gel with a linear gradient of 8.5% to 14% Polyacrylamide, and a 2.5 cm top layer of 5% acrylamide stacking gel. Five ml of 1% agarose in sodium dodecyl sulphate buffer was poured over the cylindrical gel and allowed to solidify. Electrophoresis in the second dimension was carried out towards the anode in a vertical gel apparatus at 100 V for 6.5 h, using 0.025 M Tris-HCl in 0·192 M glycine and 0.1% sodium dodecyl sulphate at pH 8·3 as the electrode solution.

The gels were fixed overnight in 50% methanol and 10% acetic acid. The fixed gels were expanded to original size in 7% acetic acid for 4 h, and dried under evacuaion. Dried gels were radiographed on Kodak Medical X-ray film in dry chambers. The usual exposure time for a gel loaded with 8 r 10^5 dpm was three weeks.

Labelling glycoproteins with iodinated Concanavalin-A

Concanavalin-A was labelled with [^{125}I], using chloramine-T as described by Hunter and Greenwood (1962). Iodinated Con-A was purified on a 8 r 0.9 cm column of

Sephadex G-75 by washing with 0.2 N acetic acid, neutralized with 1 M Tris-HCl buffer (pH 8) and stored at –40°C. The specific activity of labelled Con-A was 0.1 m C*i*/mg.

Fixed gels of brain or body wall extracts (unlabelled) were washed with distilled water giving three changes for 1 h each time and equilibrated with buffer containing 0.1 M Tris HCl pH 7,1 mM $MnCl_2$ and 1 mM $CaCl_2$. Each gel was soaked in 100 ml of fresh buffer containing 4 r 10_7 d pm of [^{125}I] labelled Con-A and 1 mg/ml of cyto-chromec for 24 h (Burridge, 1976). After repeated washing with buffer these gels were dried and radiographed.

[^{35}S]-*labelled yeast*

Saccharomyces cerevisiae was labelled according to the procedure of Graham and Stanley (1972). Ten mCi of $Na_2{}^{35}SO_4$ was added to a 20 ml culture of growing yeast in a sulphur-free medium at a cell density of 3 r 10^6/ml. After 18 h the labelled cells were centrifuged, washed thrice with sterile saline, and resuspended in 3.5 ml saline. The level of radioactivity in the suspension was about 6 r 10^9 dpm/ml.

Results

The two dimensional gel patterns of peptides from brain and body wall of 3rd instar larvae of *Drosophila* are shown in Figure 1. The larvae were labelled for 88 hours and extracts were made from 10 body walls or 50-60 brains. One might, therefore, expect that differences arising from stage of development (Tissiers *et al.,* 1974; Arking, 1978) would be greatly reduced. There is, nevertheless, a certain amount of variability in these gels. A part of this variation undoubtedly arises from the fact that the detection of faint spots near the threshold of visibility greatly depends upon the extent of exposure. In addition, there is some inescapable variation in the technique of electrophoresis itself (O Farrell, 1975: Rodgers and Shearn, 1977). In order to make a comparison of wild type and mutant patterns more reliable, we have attempted an objective assessment of the reproducibility of total protein patterns by comparing independent gels to construct idealized maps for peptides of body wall and brain.

Comparison of brain and body wall gels

The autoradiographs were enlarged to 10″ r 12″ prints. Using prominent peptides as landmarks, the entire gel was subdivided into six segments and the spots were arbitrarily numbered. One gel, each of brain and body wall from three independent experiments were then carefully compared for the presence or absence of each numbered spot and the results were tabulated in serial order. In spite of some variations in size and intensity of spots in different gels, the ‘positional identity’ of a spot could be determined with the help of surrounding landmarks. Thus, spots having the same ‘relative position’ in the different gels of brain and body wall, carried the same numbers. The overall reproducibility of spots in the brain and body wall gels may be gauged from table 1.

The body wall profile was then compared with the brain profile and the spots were classified into three groups: a) spots common to brain and body wall, b) spots unique to brain and c) spots unique to body wall. Spots that had the same ‘relative positions’ in at least two out of three gels of both brain and body wall, were called common spots. These are shown in figure 2. Two additional gels of brain and body

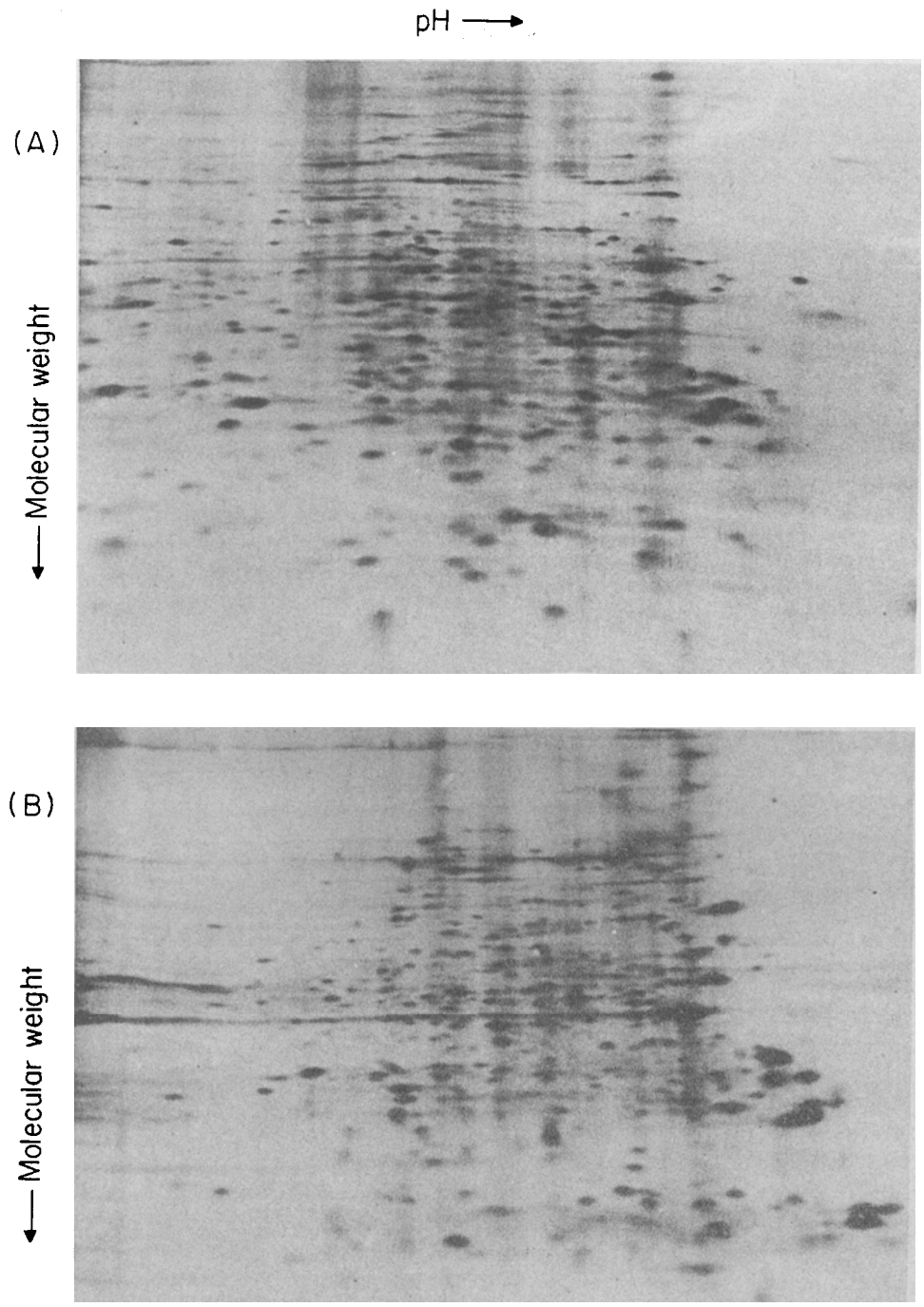

Figure 1. Two dimensional gels of [^{35}S]-labelled proteins of *Drosophila melanogaster* larvae: A, proteins of brain. B, proteins of body wall. Molecular weights and pH were estimated in parallel runs. These are to be taken as approximate. Approximately 8×10^5 dpm of [^{35}S]-counts equivalent to about 25 μ g. protein was loaded on each gel. The gel was exposed to X-ray film for 3 weeks.

Table 1. The reproducibility of peptide spots in brain and body wall two dimensional gels.

	Brain	Body wall
Spots present in all 3 gels	520	514
Spots present in 2 gels out of three	91	105
Total	611	619
Spots present in 1 gel out of three	39	27

Only spots present in at least two gels out of three, are considered reproducible. Spots present in just one gel are considered irreproducible, and are not included in the total.

wall were examined for unique spots. We have considered a spot unique to either brain or body wall, if it was seen in at least three out of five gels of one kind and none of the other. The distribution of brain specific and body wall specific spots is shown in figure 3. The differences between these tissues in selected segments of the gels are presented in figure 4. Out of the total of about 620 spots recorded, 66 were unique to body wall and 61 unique to brain. About 80% of the spots were common to both tissues.

Table 2. Reproducibility of glycoprotein spots in two dimensional gels.

	Brain	Body wall
Spots present in all 3 gels	30	16
Spots present in 2 gels out of 3	14	25
Total	44	41
Spots present in 1 gel out of 3	6	8

Spots present in just 1 gel are not included in the total as they are considered irreproducible.

Glycoproteins

Concanavalin-A binding glycoproteins were visualized by soaking two dimensional gels of extracts of brain and body wall of unlabelled larvae in con-A labelled with ^{125} I. Glycoprotein spots were compared in the same manner as total proteins. Three gels each of body wall and brain were examined and spots present in two out of three gels were considered reproducible. These spots were classified as common and unique. Common spots were present in at least 2 out of 3 gels of both brain and body wall. Unique spots were present in at least 2 out of three gels of one kind and none of the other. The reproducibility of glycoprotein spots is presented in Table 2 and the distribution of common and unique spots is shown in Figure 5. The body wall extracts had 41 spots of which 8 were unique. In brain, 8 out of 44 spots were unique. Twenty-seven spots appeared to be common to both the tissues.

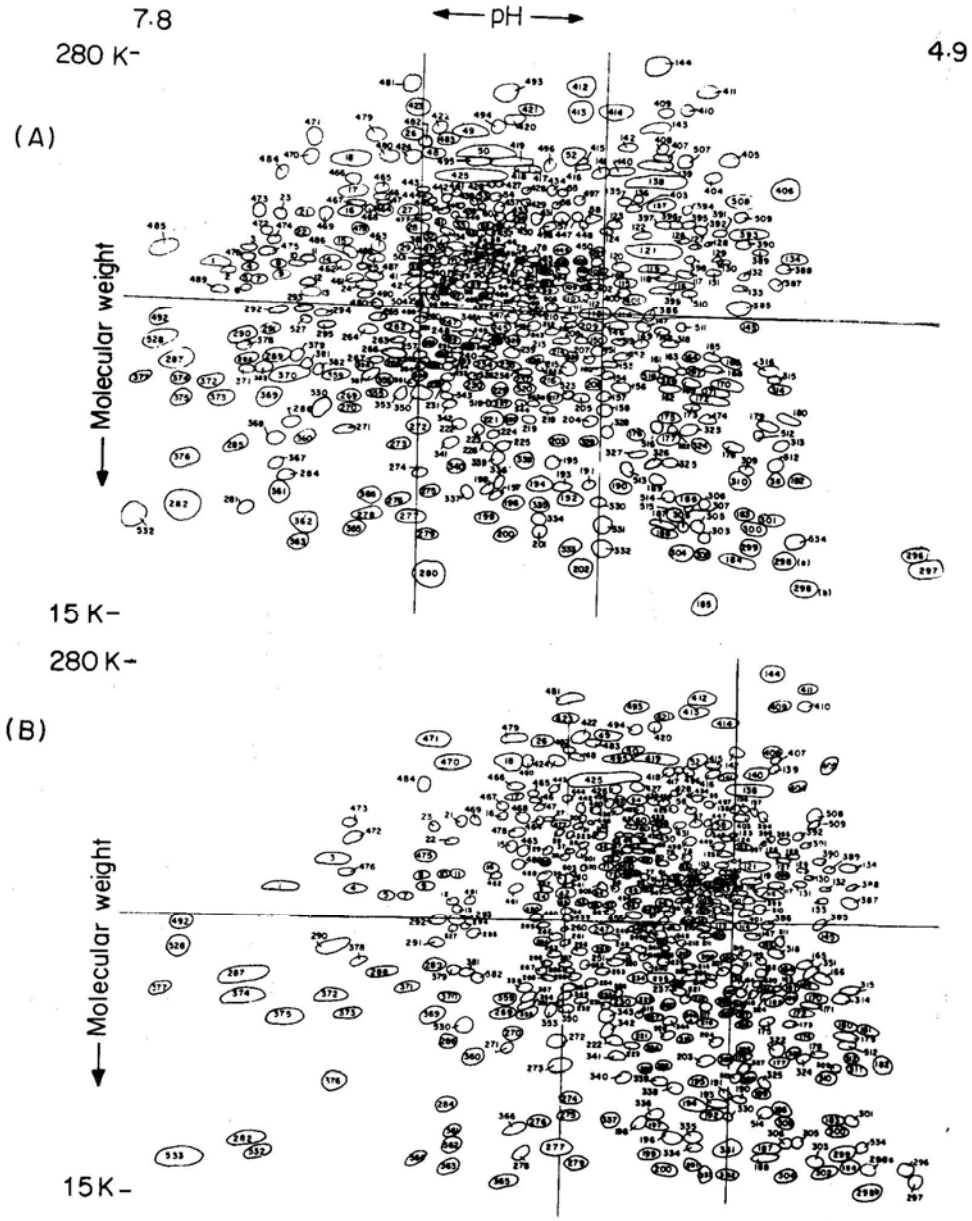

Figure 2. Tracings of radioautographs for comparing two dimensional gels: For convenience of comparison the radioautograph is divided into six segments. Spots common to brain and body wall were circled and numbered arbitrarily from 1 to 534. A, brain; B, body wall. The total number of common spots is 517 as 17 spots initially numbered turned out to be uncommon. Corresponding spots from brain and body wall carry identical numbers. A common spot is not necessarily present in every gel eg. spot 406 and 485 above (see text for definition of common spots).

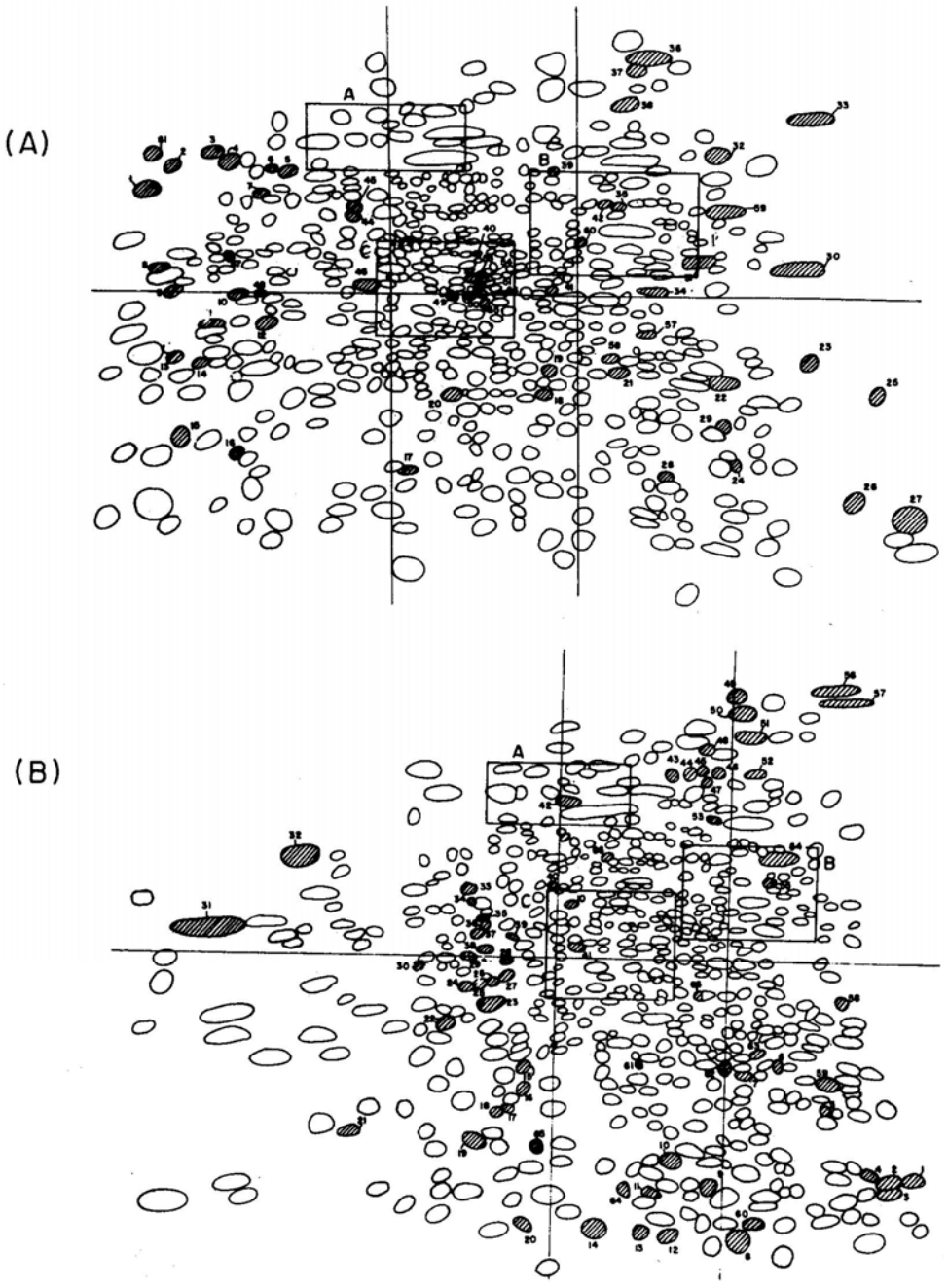

Figure 3. Distribution of spots unique to brain and body wall: A-brain and B-body wall. 61 unique spots in the brain gel and 66 unique spots in the body wall gel are cross hatched. Segments of which enlargements have been shown in Figure 4 are marked as insets.

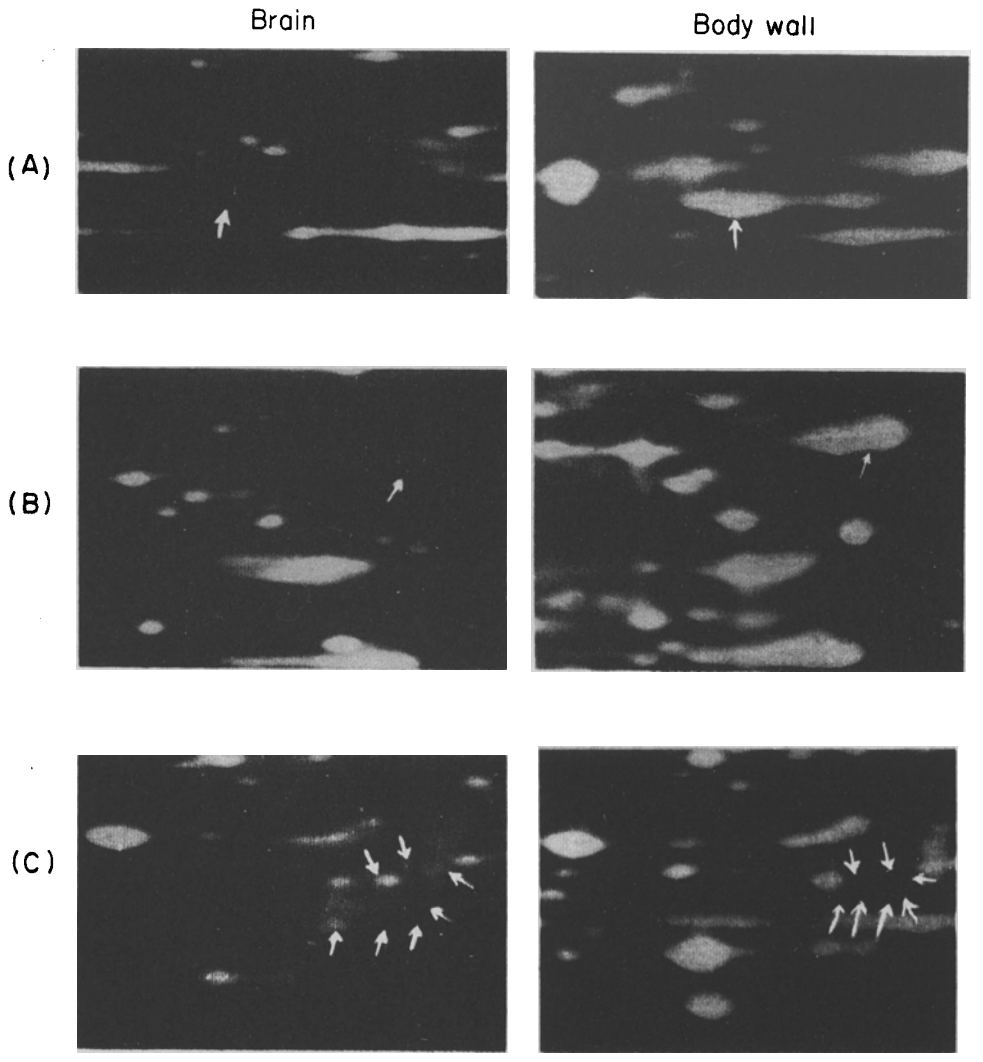

Figure 4. Segmental enlargements of selected regions of brain and body wall gels showing the unique spots: Arrows show the presence of a unique spot in gels of one type and its absence in a corresponding position in gels of the other type. A, B, and C represent the segments of brain and body wall gels which are shown as insets in Figure 3.

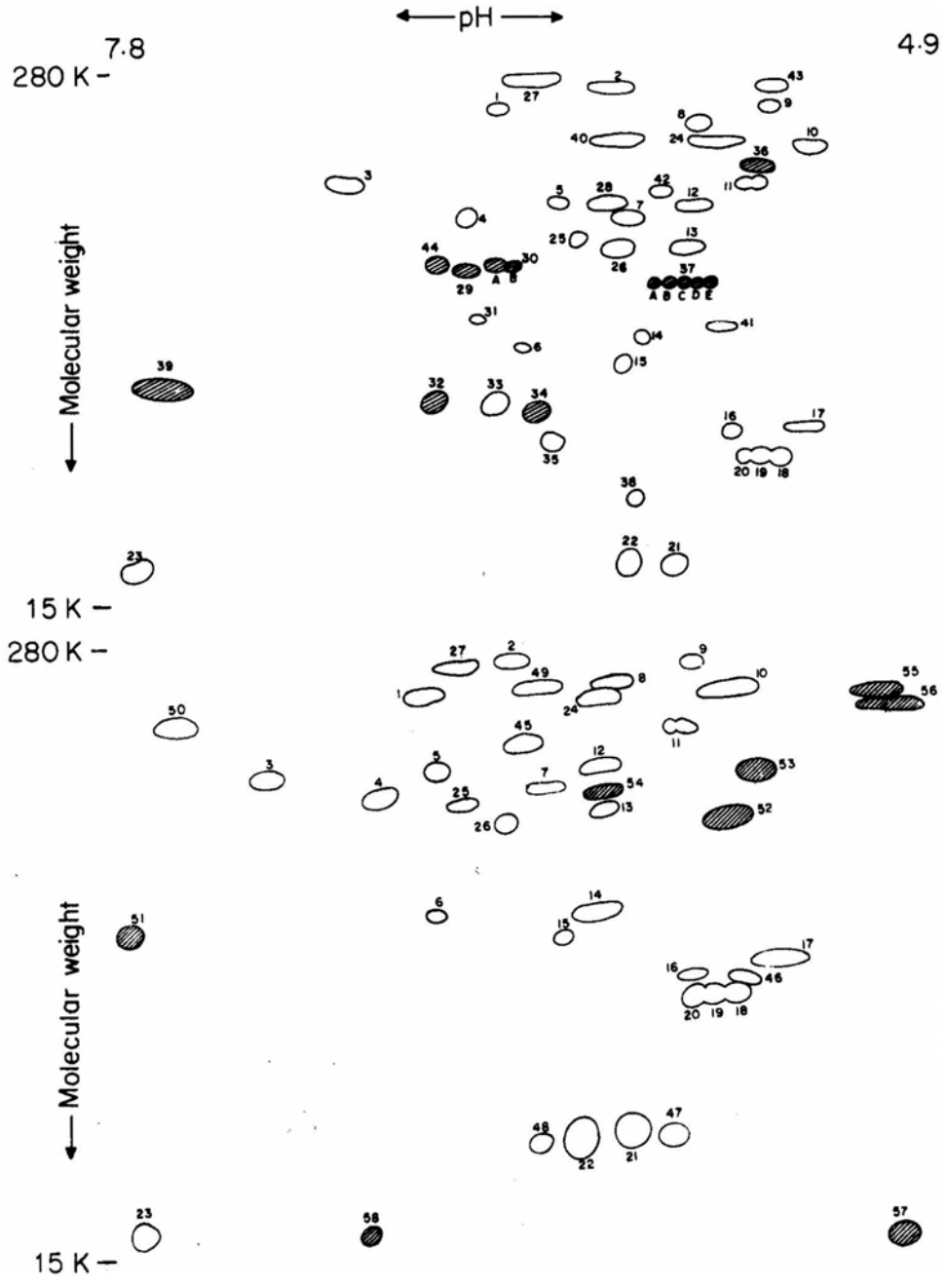

Figure 5. Tracings of radioautographs showing Con-A binding glycoproteins in 2D gels: A-brain B-body wall. Common spots are numbered 1-27. Unique spots are cross hatched.

Discussion

O'Farrel (1975) was able to detect over *1100* spots on two dimensional gels of *Escherchia coli* and similar numbers in *Caenorhabditis elegans.* The observed numbers were close to the estimated number of polypeptides in these organisms. Rodgers and Shearn (1977) found 330 spots in imaginal discs of *Drosophila* and 435 spots in whole larvae devoid of discs, labelled for short period. The 600 or so spots seen by us in uniformly labelled larval tissues are considerably short of the total number of sodium dodecyl sulphate peptides that might be expected on theoretical grounds (Judd *et al.*, 1972). This is not surprising. As pointed out by Rodgers and Shearn (1977), the technique used by us will fail to detect peptides which are insoluble in 9 M urea and NP40, peptides deficient in sulphur, peptides whose molecular weights lie beyond the range of 15,000-280,000 daltons or whose isoelectric points are beyond the chosen range of pH. Besides we have neglected extremely faint as well as erratic spots. The observed number of spots is nevertheless large enough to make the arduous task of comparing wild type and mutant patterns worthwhile.

A comparison of body wall and brain peptides allows us to estimate the proportion of tissue specific proteins. The 66 unique spots in body wall extracts and 61 in brain represent about 10% of the total. Although the proportion of tissue specific proteins in the two cases appears to be the same, the distribution of the spots across the gel is noticeably distinctive. The unique spots in brain gels are, more or less, uniformly distributed over the entire gel. The unique spots in body wall gels, on the other hand are clustered towards the alkaline end of the gel in the pI range of 7-7·5. It is likely that muscle specific proteins are among these spots.

Con-A-binding glycoproteins make up about 7% of larval proteins, and 64% of the glycoprotein spots are common to body wall and brain. 18% of brain glycoproteins (8 out of 44) and 18% of body wall glycoproteins (8 out of 41) are unique. The proportion of tissue specific spots among glycoproteins, thus appears to be higher than other proteins.

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