Developmental Regulation of *Dictyostelium discoideum* Plasma Membrane Proteins

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ABSTRACT Developmental changes in the plasma membrane proteins of *Dictyostelium discoideum* have been studied using metabolic labeling with [^{35}S]methionine and two-dimensional electrophoresis. Pulse labeling for 1 h at the early interphase, late interphase, aggregation, and tip formation stages of development showed that the profile of newly synthesized plasma membrane proteins changed dramatically over this interval. Only 14% of the polypeptide species were synthesized at all four stages at detectable levels; 86% of the species changed over this developmental interval according to the criterion that they were synthesized at some but not all of the four stages tested. Long-term labeling during vegetative growth followed by initiation of development showed that the "steady-state" levels of the plasma membrane proteins changed very little over the same period. The only changes were in minor species (33% overall change). Similar analyses of whole cell proteins showed 27 and 20% change, respectively. Cell surface radioiodination revealed 52 external proteins in the plasma membrane. Comparison with the uniform methionine labeling results showed that these proteins were, with one notable exception, minor membrane components. In these external proteins, also, developmental changes were limited and were observed in the less abundant species.

These results demonstrate the existence of two general classes of plasma membrane proteins. The first is a population of high-abundance proteins that are present in vegetative cells and are largely conserved through development. These possibly serve "housekeeping" functions common to all stages. The second class consists of low-abundance species that are expressed in a highly stage-specific manner and which presumably participate in developmentally important functions.

During the course of developmental processes, the plasma membrane is a likely target of extensive biochemical differentiation since it mediates the interaction and communication between the cell and its environment. In many developmental systems it is difficult to investigate developmental changes in the plasma membrane since a number of different cell types may be generated simultaneously. The cellular slime mold *Dictyostelium discoideum* is a good model system for study of this issue since, during early development, the amoebae differentiate synchronously and give rise to a chemotactically responsive, mutually cohesive population. The amoebae then aggregate into a tissue-like cell mass from which an organizing tip emerges. At this stage, or soon thereafter, two cell types are generated that are the precursors of the stalk and spore cells comprising the final fruiting body (reviewed in reference 1). Thus, studies on the changes in plasma membrane proteins during the developmental interval between vegetative growth and tip formation (14–15 h) should be able to provide clear information regarding the extent of plasma membrane protein alterations during cellular differentiation without the complications introduced by generation of multiple cell types.

Using two-dimensional electrophoretic analysis, Alton and Lodish (2) showed that there is a 25% change in the profiles of newly synthesized whole cell proteins over the total 24-h developmental time course. Several laboratories have investigated the developmental regulation of *D. discoideum* plasma membrane proteins. Developmental changes in cell surface proteins (3, 4), lectin binding proteins (5, 6), glycoproteins (7–12) as well as total plasma membrane proteins (4, 8–13) have been studied using one-dimensional electrophoresis. Although the data cannot always be directly compared due to variations in the techniques used (e.g., membrane isolation procedures, electrophoresis conditions, detection methods), the general result that emerges is that there are relatively fewer

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changes in the protein composition of the plasma membrane than anticipated, the major changes being observed in cell surface glycoproteins. One possible reason for the limited extent of change observed by previous investigators could be the restricted resolving power of one-dimensional electrophoresis. This interpretation is supported by the recent results of Toda et al. (10) and Ono et al. (13) who have shown, using two-dimensional electrophoresis, that there are more extensive changes in cell surface glycoproteins and plasma membrane proteins during early differentiation of *D. discoideum* than previously observed. Employing a newly devised method for isolation of 30-fold purified plasma membranes from this organism (14), we have reinvestigated the developmental regulation of plasma membrane proteins using two-dimensional electrophoretic separation (15). Metabolic labeling with [^{35}S]-methionine was used to identify proteins. Two labeling regimens were followed: pulse labeling to identify newly synthesized proteins at four developmental stages, and long-term labeling to detect changes in the absolute levels of individual species at each stage. The emphasis was on analysis of plasma membrane proteins, but concurrent studies were performed on total cellular proteins against the background of the report cited above (2). In addition, we have studied developmental changes in external plasma membrane proteins using cell surface labeling with ^{125}I.

MATERIALS AND METHODS

Strains and Growth Conditions: *D. discoideum* strain A3 was grown in shaken suspension (120 rpm) at 22°C in HL-5 broth (16).

Incorporation of [^{35}S]Methionine: Cells were harvested during late exponential growth at 5–8 × 10^6/ml by centrifugation at 500 *g* for 5 min. They were washed and resuspended in KPM buffer (20 mM potassium phosphate, 2 mM $MgSO_4$, pH 6.1). Pulse labeling was performed according to a modification of the Alton and Lodish (2) procedure. Amoebae (1 ml, 1 × 10^8 amoebae) were dispersed on 47-mm Nuclepore filters (0.5-μm pore size; Nuclepore Corp., Pleasanton, CA) resting on 2% Agar in KPM. The petri dish was left open for 30 min to allow evaporation. Under these conditions, streaming was obvious by 7–8 h, loose mounds formed at 8–9 h, and tips appeared at 13–14 h. At appropriate times the filters were transferred with forceps to a 10–30-μl droplet containing 75–100 μCi of [^{35}S]methionine in an otherwise empty petri dish, and the dish was covered with wet filter paper. Cells were harvested after 1 h of labeling and washed with ice-cold KPM.

For uniform labeling during vegetative growth, amoebae at 5–8 × 10^6/ml in HL-5 were diluted 4-fold into HL-5 containing [^{35}S]methionine (Amersham Corp., Arlington Heights, IL, at least 500 Ci/mmol) at 50–100 μCi/ml. Cysteine at 2 mM was included to prevent possible incorporation after metabolic conversion of the methionine. After growth to 5–8 × 10^6 amoebae/ml (two generations) the culture was harvested and plated on 2% KPM Agar containing 2 mM cysteine. These treatments did not alter the normal times or patterns of morphogenesis. Cells were harvested at appropriate times and plasma membranes were prepared.

Membrane Preparation: Cells were washed and resuspended at 3–5 × 10^7/ml in lysis buffer (5 mM glycine, pH 8.5 at 25°C, pH 8.95 at 2°C, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$) and allowed to warm to room temperature briefly (2–4 min). A 5-μm pore size Nuclepore polycarbonate filter was placed in the appropriate holder (Swin-Lok holder, Nuclepore Corp.), and the holder was attached to a syringe. The cells were placed in the syringe and lysed by forcing them through the filter. Lysis was always over 95%, usually complete. However, when small numbers of cells are used (<1 × 10^8), lysis is <95% complete. To avoid this problem, filters were "activated" by passage of 3–5 × 10^8 unlabeled cells and subsequent rising with buffer prior to lysis of the small sample. The lysate was immediately chilled in an ice bath and subjected to centrifugation at 5,000 *g* for 20 min. The crude membrane pellet was resuspended in a minimal volume of lysis buffer and layered over a 15-ml linear sucrose gradient (0.75–1.5 M sucrose in 50 mM glycine, pH 8.5 at 22°C) with a cushion of 2.5 M sucrose in 50 mM glycine. The gradient was centrifuged for 18 h at 100,000 *g* at 2°C. Plasma membranes form a triplet of bands in the middle of the gradient and were collected together by lateral puncture of the gradient tube. The fractions were diluted with 5–10 vol of 20 mM Tris-HCl buffer (pH 7.4) and harvested by centrifugation at 20,000 *g* for 90 min. This procedure yields 35–50% of the cellular plasma membrane which is 30-fold purified on the basis of yield and enrichment of membrane-impermeable, covalent cell surface radiolabels. The preparation shows minimal contamination by lysosomes and mitochondria (<1%) and endoplasmic reticulum (<2%) as determined by marker enzyme assays. Full details of the lysis procedure and characterization of the plasma membranes will be reported elsewhere (14). Since relatively small numbers of cells were used for metabolic labeling, the membrane bands could not be detected visually in the sucrose gradients. Therefore, for each gradient containing labeled membranes, an identical gradient was run in parallel containing material from 1.5 × 10^9 unlabeled cells at the same developmental stage. Visual comparison of the two gradients was used to identify the region containing the radioactive plasma membranes. The protein load on the gradient does not alter the equilibrium position of the plasma membrane bands as monitored by protein profiles on two-dimensional gels stained with Coomassie Blue (data not shown).

Iodo-Gen-catalyzed Radioiodination: Amoebae were starved on 2% KPM Agar at 1 × 10^8 cells per plate. Cells at each developmental stage were harvested, washed with 50 mM glycine (pH 8.5) after gentle vortexing to disrupt cell clumps, and then washed and resuspended in 2 ml of KPM for radioiodination (17).

Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycouril obtained from Pierce Chemical Co., Rockford, IL) was dissolved in chloroform at 100 μg/ml, and 200 μl were added to a scintillation vial and dispersed on the vial walls by gentle vortex agitation. Solvent was removed under a stream of nitrogen, and the vial was stored at room temperature in a desiccator. Just prior to use, the vial was rinsed twice with 2-ml portions of KPM to remove any loose flakes of Iodo-Gen. Cells (1 × 10^8) were added to the vial, and iodination was initiated immediately by the addition of 1 mCi of ^{125}I (carrier and reductant free, New England Nuclear, Boston, MA). The vial was incubated at room temperature for 20 min with occasional swirling. The reaction was quenched by addition of a solution of NaI to 2 mM. The cells were removed from the vial, washed four times with KPM containing 1 mM NaI, and lysed as described, except that the lysis buffer contained 1 mM NaI. After centrifugation of the lysate for 20 min at 5,000 *g*, the pellet was solubilized for electrophoresis.

SDS Gel Electrophoresis: Harvested membrane pellets were drained and resuspended in 15% glycerol, 5% β-mercaptoethanol, 50 mM Tris-HCl, pH 6.8, with vigorous vortex mixing, and 10% SDS was added to a final concentration of 3%. The samples were heated in a boiling water bath for 3 min. Slabs of 10% polyacrylamide, with 4% stacking gels, were run in 0.1% SDS using the Ornstein-Davis stacking system (18). The gels were stained with Coomassie Blue (19), photographed with Polaroid type 55 film using a yellow filter, dried, and exposed to Kodak XR-5 film for autoradiography.

Two-dimensional Gel Electrophoresis: This was performed with modifications of the methods of Ames and Nikaido (20) and O'Farrell (15). Gradient fractions were diluted and sedimented as described above. The pellet was resuspended in 40 μl of isoelectric focusing sample buffer (10 mM Tris-HCl, pH 6.8, 20% glycerol, 10% β-mercaptoethanol) with vigorous vortex mixing, and 10 μl of 10% SDS were added. The samples were heated for 3 min in a boiling water bath, chilled, and diluted with 50 μl of dilution buffer (16% Nonidet P-40, 9.5 M urea, 7.5% β-mercaptoethanol, and 3% of a cocktail containing ampholytes of the pH ranges 4–6, 6–8, and 3.5–10 in the ratio 2:2:1). Each sample was saturated with crystals of urea and loaded into the isoelectric focusing tubes. <50 μg of protein was loaded per tube since larger amounts led to streaking of the relatively insoluble membrane proteins. Under these conditions, at least 95% of the radioactivity from [^{35}S]methionine-labeled membranes was found to enter the gel. Other conditions for electrophoresis were as described (15), except that the ampholytes used in the gel were of the pH ranges 3–10, 4–6, and 7–9 in the ratio 7:3:3.

The gels were run at 500 V for 18 h at room temperature and extruded into curved spatulas. To each spatula, 1 ml of SDS buffer (2% SDS, 25 mM Tris, pH 6.8, 5% β-mercaptoethanol) was added and the gel and buffer on the spatula were frozen at −70°C. The final pH gradient in the isoelectric focusing gel was linear from pH 4 to pH 7 with a trailing, shallow gradient up to pH 7.5 over the last 20% of the gel.

The second-dimension electrophoresis was in 10% polyacrylamide slab gels with 4% stacking gels containing 0.1% SDS. Prior to loading on the second dimension, ~1 cm of the acid end of the isoelectric focusing gel was cut off and discarded. This contains a plug of SDS which interferes with stacking and has no protein. The isoelectric focusing gels were sealed in place with 0.5% Agar containing 1% SDS. Molecular weight standards were loaded in slots cut at the edge of the agarose sealing gel. The slabs were run at 15 mA until the dye front entered the running gel and at 30–40 mA thereafter.

For fluorography, gels were impregnated with 2,5-diphenyloxazole (PPO; Amersham Corp., Arlington Heights, IL) as described by Laskey and Mills (21), dried, and exposed at −70°C to Kodak XR-5 film which was pre-exposed to an

absorbance level of 0.4 OD. Gel exposure times were chosen for optimal resolution of the sample type rather than identical overall intensity, i.e., membrane proteins streak more severely than whole cell samples.

Protein Determination: Protein was assayed according to the method of Bradford (22) as described in Bio-Rad Laboratories (Richmond, CA) Bulletin no. 1051.

Materials: Carrier-free ^{125}I was obtained from New England Nuclear. Nuclepore filters and Swin-Lok holders were purchased from VWR Scientific, Inc. (San Francisco, CA). NP-40 was obtained from Shell Chemical Co. (Houston, TX), ampholytes of 3–10 pH range from Bio-Rad Laboratories, and narrow-range ampholytes from LKB Instruments, Inc. (Gaithersburg, MD). Electrophoresis reagents were all obtained from Bio-Rad Laboratories except urea which was obtained from EM Chemicals (Hawthorne, NY). Enzyme grade sucrose was obtained from Becton-Dickinson & Co., Schwartz-Mann Div. (Oxnard, CA). All other chemicals were of reagent grade.

RESULTS

Synthesis of Plasma Membrane Proteins

To identify the newly synthesized plasma membrane proteins at each developmental stage, pulse labeling with [^{35}S]-methionine was used. Initial experiments were performed to establish optimal labeling conditions. The use of Nuclepore filters as a support gave eight- to ninefold greater incorporation of added [^{35}S]methionine into protein than use of filter paper as described by others (2), presumably because Nuclepore filters are very thin (10–15 μm) and thus do not trap or dilute the radiolabel in the droplet. When labeling was performed at three developmental stages for 30 and 60 min, the isolated plasma membranes contained, respectively, 0.15 and 1.0% of the radioactivity incorporated into total cellular proteins. This suggested that 60 min were required for the majority of newly synthesized proteins to reach the plasma membrane, an interpretation supported by pulse-chase experiments (not shown).

Four stages in the developmental program were chosen for study: early interphase, late interphase, aggregation, and tip formation. After and possibly during tip emergence, there are two distinct cell types that have been shown to have differences in their plasma membrane composition (23). The labeling schedule for early and late interphase was 0.5–1.5 h and 3.5–4.5 h poststarvation. For the other stages, initiation of labeling was determined on the basis of visual observations since the precise temporal appearance of these stages is highly dependent on environmental factors such as humidity, temperature, etc. The actual initiation of labeling was always within 8.5 ± 1 h for the aggregation stage and 13.5 ± 1 h for the tip formation stage.

Plasma membranes were prepared and fractionated on two-dimensional gels. Fig. 1, *a–d* shows fluorograms of gels corresponding to early and late interphase, aggregation, and tip formation, respectively. Superficial inspection of Fig. 1 shows that the observed components are dramatically different and

TABLE I

Developmental Changes of Plasma Membrane Proteins Detected by Pulse Labeling

Stages compared	Total no. of spots detected	Common spots	Percentage change
Early—late interphase	155	73	53
Late interphase—aggregation	149	55	63
Aggregation—tip formation	150	38	75
Early interphase—tip formation	183	26	86

Pairs of fluorograms from Fig. 1 were compared by superimposition and spots were scored visually as present or absent. Species that changed only in intensity were scored as common spots.

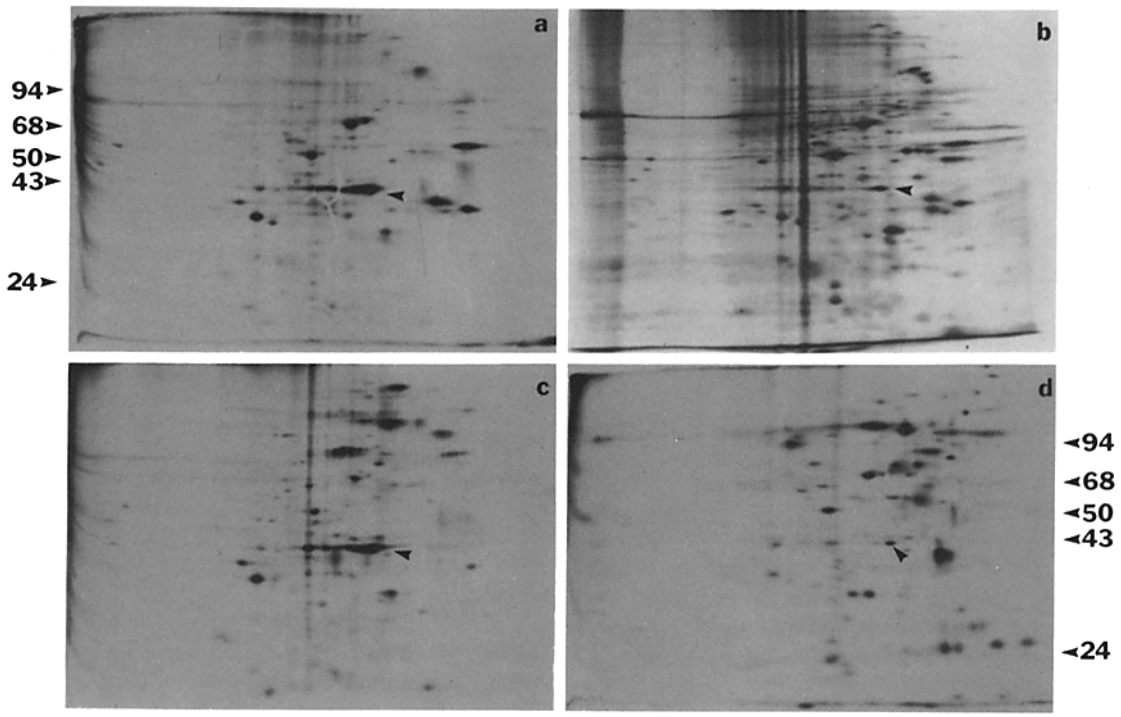


FIGURE 1 Fluorograms of pulse-labeled plasma membrane proteins. Plasma membranes were isolated from pulse-labeled cells and fractionated on two-dimensional gels that were subjected to fluorography as described in Materials and Methods. (*a–d*) Fluorograms obtained from early and late interphase, aggregation, and tip formation stages, respectively. For the gels corresponding to *a*, *c*, and *d*, 100,000 cpm of protein-bound ^{35}S was loaded. For the gel corresponding to *d*, 150,000 cpm was loaded. All exposures were for 20 d. Isoelectric focusing was in the horizontal dimension, with the acidic end to the right in each panel. SDS PAGE was in the vertical dimension. Arrowheads indicate the spot corresponding to actin.

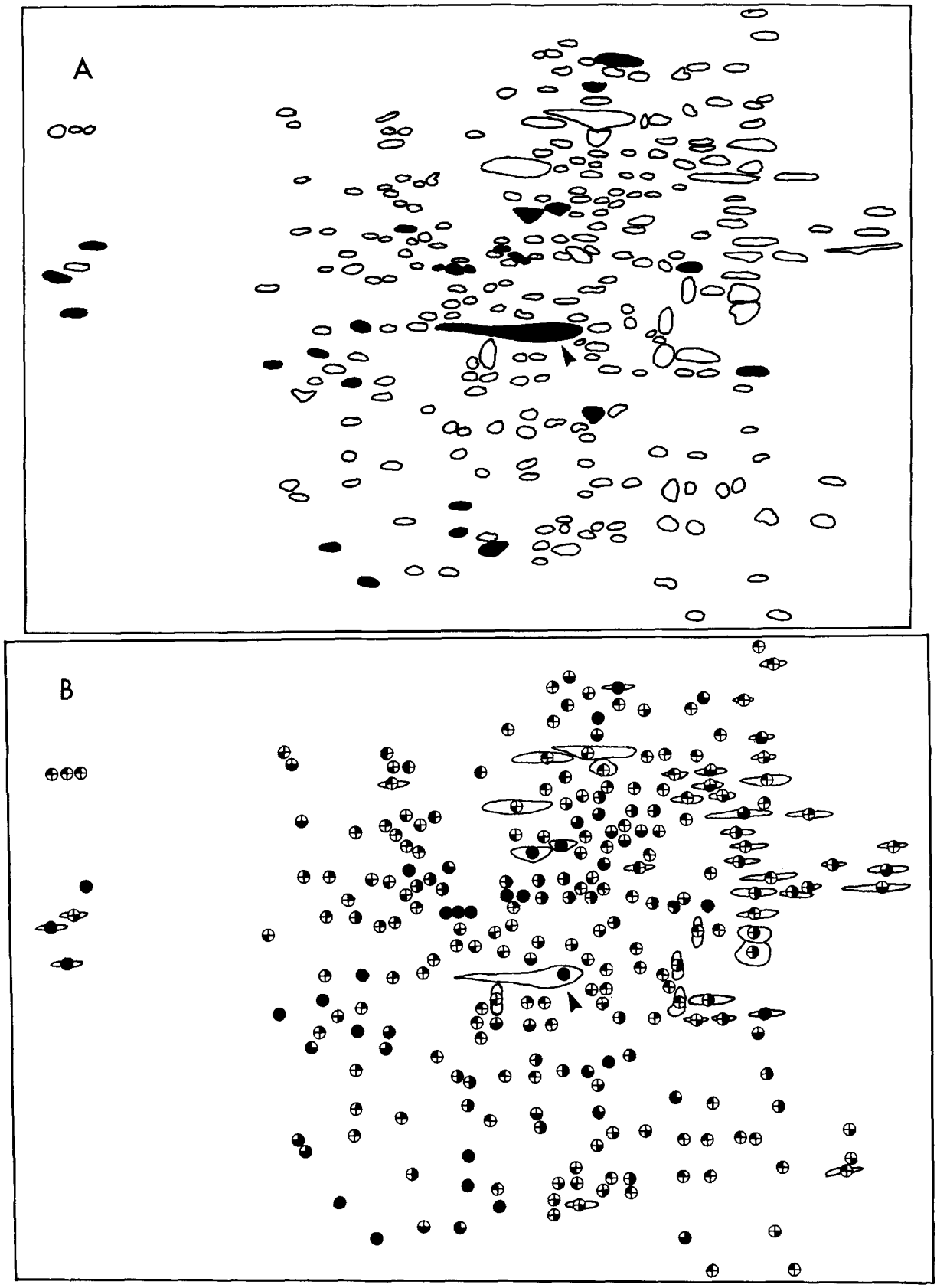


FIGURE 2 Composites of pulse-labeled plasma membrane protein gels. In *A*, the fluorograms of Fig. 1 were superimposed and compared. All the spots in the four panels of Fig. 1 are represented in this composite which has been drawn so that the spot shapes approximate those on the originals. Species that are found in all the four gels are represented by filled spots. Actin is indicated by an arrowhead. In *B*, each spot of *A* is represented by a circle. In some cases, characteristics of the original spots have been drawn around the circles to facilitate comparison. Each circle is divided into four quadrants. The quadrants that are filled represent the particular stage at which the given species is detected, according to the following scheme. *Right upper*, early interphase; *right lower*, late interphase; *left lower*, aggregation; *left upper*, tip formation.

suggests that most of the proteins are highly stage-specific in their expression. A detailed comparison supports this conclusion. Pairs of fluorograms were superimposed on a light box, and spots were scored manually as being present in both or only one. Entire fluorograms are not exactly superimposable, but there are sufficient common spots that regional patterns are superimposable and can be compared. Spots directly on vertical streaks were omitted in this analysis, and stuttering spots in the horizontal dimension were scored as a single species. Also, all gels were independently analyzed by both authors with essentially identical results.

The results of these comparisons are shown in Table I. From early to late interphase, 155 spots can be discerned on the two gels; of these spots, 73 are in common, 37 are found only at the early stage, and 45 new ones appear at the later stage. This corresponds to a change of 53%, that is, 53% of the species were stage specific. Similarly, there is a change of 63% from late interphase to aggregation. The largest change, however, is between the aggregation and tip formation stages, namely 75%. The total change from early interphase to tip formation is 86%, with only 26 of the total 183 spots on both gels being in common. In this analysis, only disappearance or appearance of spots was considered as change; a number of proteins common to each pair of gels differ in intensity and therefore change in relative levels of synthesis from one stage to another. A striking example is actin, indicated by arrowheads on the fluorograms. If proteins that vary in amount were included, the percentage change would be close to 100%.

Fig. 2, *a* and *b* shows two different types of composites derived from the fluorograms in Fig. 1. Fig. 2*a* represents the total collection of proteins from all four gels, drawn so that the spot shapes approximate the originals. Proteins that are found in all four gels are shown as filled spots. Fig. 2*b* is a schematic composite, with each protein being represented by a circle divided into four quadrants. The particular quadrant filled represents the time at which the given protein is synthesized, and clockwise, from the right upper through left upper, the quadrants correspond to early interphase through tip formation. Fig. 2*b* also demonstrates that many of the proteins are highly stage specific in that they are found at only one of the four stages considered. Detection of these changes required two-dimensional electrophoretic analysis. When the

TABLE II

Developmental Changes of Plasma Membrane Proteins Detected by Uniform Labeling

Stages compared	Total no. of spots detected	Common spots	Percentage change
Early—late interphase	135	129	4.5
Late interphase—aggregation	137	123	10
Aggregation—tip formation	125	92	26
Early interphase—tip formation	155	104	33

Pairs of fluorograms from Fig. 3 were compared by superimposition and spots were scored visually as present or absent. Species that changed only in intensity were scored as common spots.

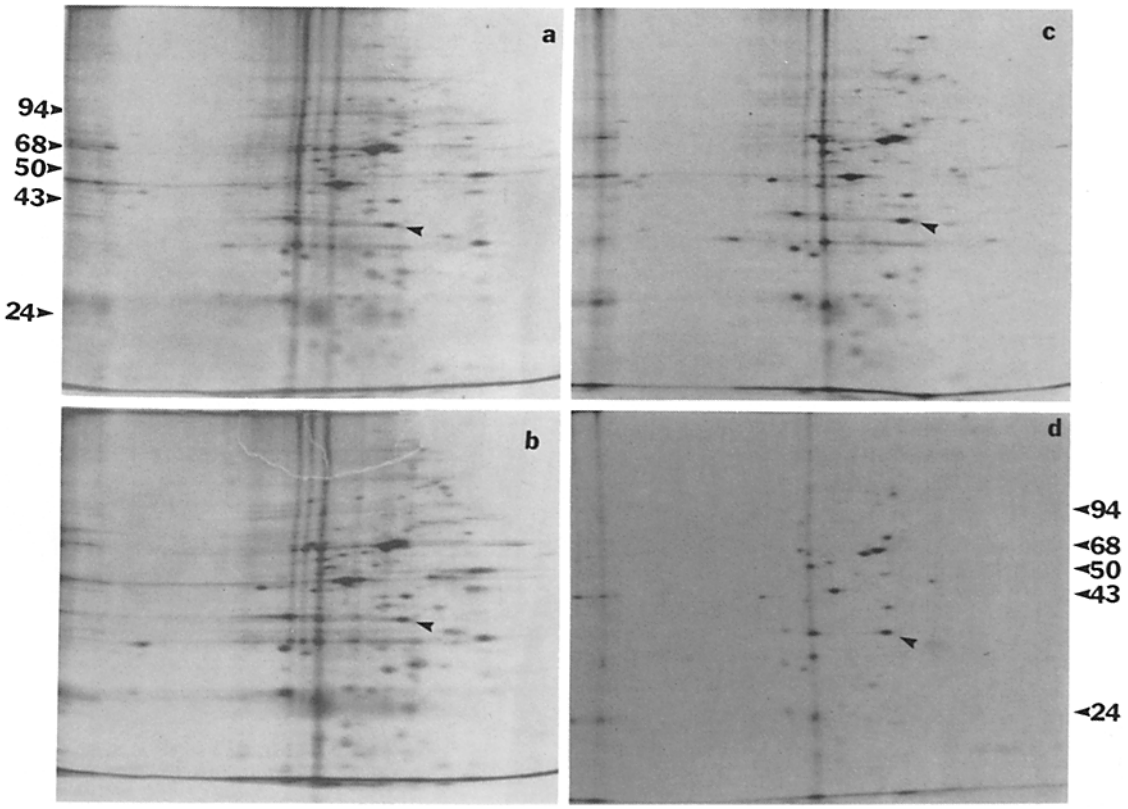


FIGURE 3 Fluorograms of uniformly labeled plasma membrane proteins. Cells were labeled for two generations of growth with [^{35}S]methionine, allowed to develop, and plasma membranes were isolated at four stages of development and electrophoresed on two-dimensional gels as described in Materials and Methods. (*a–d*) Fluorogram gels, corresponding respectively to early and late interphase, aggregation, and tip formation stages. The radioactivity loaded in the gels corresponding to *a–d* is, respectively, 130,000, 150,000, 100,000, and 90,000 cpm of protein-bound ^{35}S. All the gels were exposed for 25 d. Other details are as in Fig. 1.

same samples were fractionated on one-dimensional SDS gels (not shown) the number of changes detected was severely reduced due to overlapping or co-migration of many species that are resolved in Fig. 1.

Long-term Labeling Studies

Drastic changes in the profile of newly synthesized proteins do not imply similar changes in overall protein composition of the plasma membrane. To determine the changes in "steady-state" levels, cells were labeled with [^{35}S]methionine for two generations of growth to allow uniform labeling of all proteins, and development was induced by starvation. Growth in the presence of the isotope did not alter the time course of subsequent development. Two-dimensional gel analysis was performed on plasma membranes isolated at early and late interphase (harvested 1 and 4 h after starvation, respectively), aggregation, and tip formation (harvested as visually monitored). Fluorograms of these gels are shown in Fig. 3, *a–d*, respectively. Though the gels are not of equal quality, it is apparent on initial inspection that there is little change. Detailed comparisons, as in the previous pulse-labeled case, support this initial conclusion. The results of these comparisons are shown in Table II. From early to late interphase, there is a 4% change: 4 spots disappear and 2 new ones appear. There is a 10% change from late interphase to aggregation, and a 26% change from aggregation to tip formation. The overall change from early interphase to tip formation is 33%, significantly less than that obtained with pulse labeling. However, the maximal change is again in the aggregation to tip formation transition. In addition, most of the changes are confined to minor components.

The number of spots detected on the fluorogram corresponding to tip formation (Fig. 3*d*) is less than that for the others (85 as compared with 131, 134, and 115 for Fig. 3, *a–c*, respectively). Therefore, the percentage change from aggregation to tip formation could be considered to be artificially high. However, even in terms of appearance of new species, this developmental interval exhibits the largest change. Also, lower detection levels in one gel as compared with another would not alter the percentage change between the two, since lower levels of detection should overestimate the decrease and underestimate the increase (or vice versa), and these effects should, on the average, cancel each other.

Composites of the four gels of Fig. 3 have been constructed, as for the pulse labeling case; these are shown in Fig. 4 (p. 1552). The same system of representing developmental changes (Fig. 2) has been followed. Comparison of Figs. 2 and 4 again emphasizes the differences in behavior of the steady-state and newly synthesized protein pools. It is also notable that long-term labeling detects only 19 stage-specific species, and that, of these, 15 are specific for the tip formation stage.

Some of the new spots that appeared at various stages after long-term labeling could be seen to arise from de novo synthesis as revealed by pulse labeling. Fig. 5 shows composites derived from Figs. 1, 2 and 3, 4; spots that can be correlated are shown as filled symbols on the long-term labeling composite (Fig. 5*a*) and are darkened in the respective quadrants in the pulse-labeling composite (Fig. 5*b*). Other spots are left open. While some correlations can be made, not all new spots can be seen to arise due to increased synthesis. Possible reasons for this are considered in the Discussion.

Interestingly, most of the newly synthesized species that appear at each stage are not detectable as new spots in the long-term labeling gels. The implication of this result is that most of the newly synthesized species at each developmental stage are minor constituents that do not accumulate in the membrane to sufficient levels to be detected under conditions for identification of major membrane components.

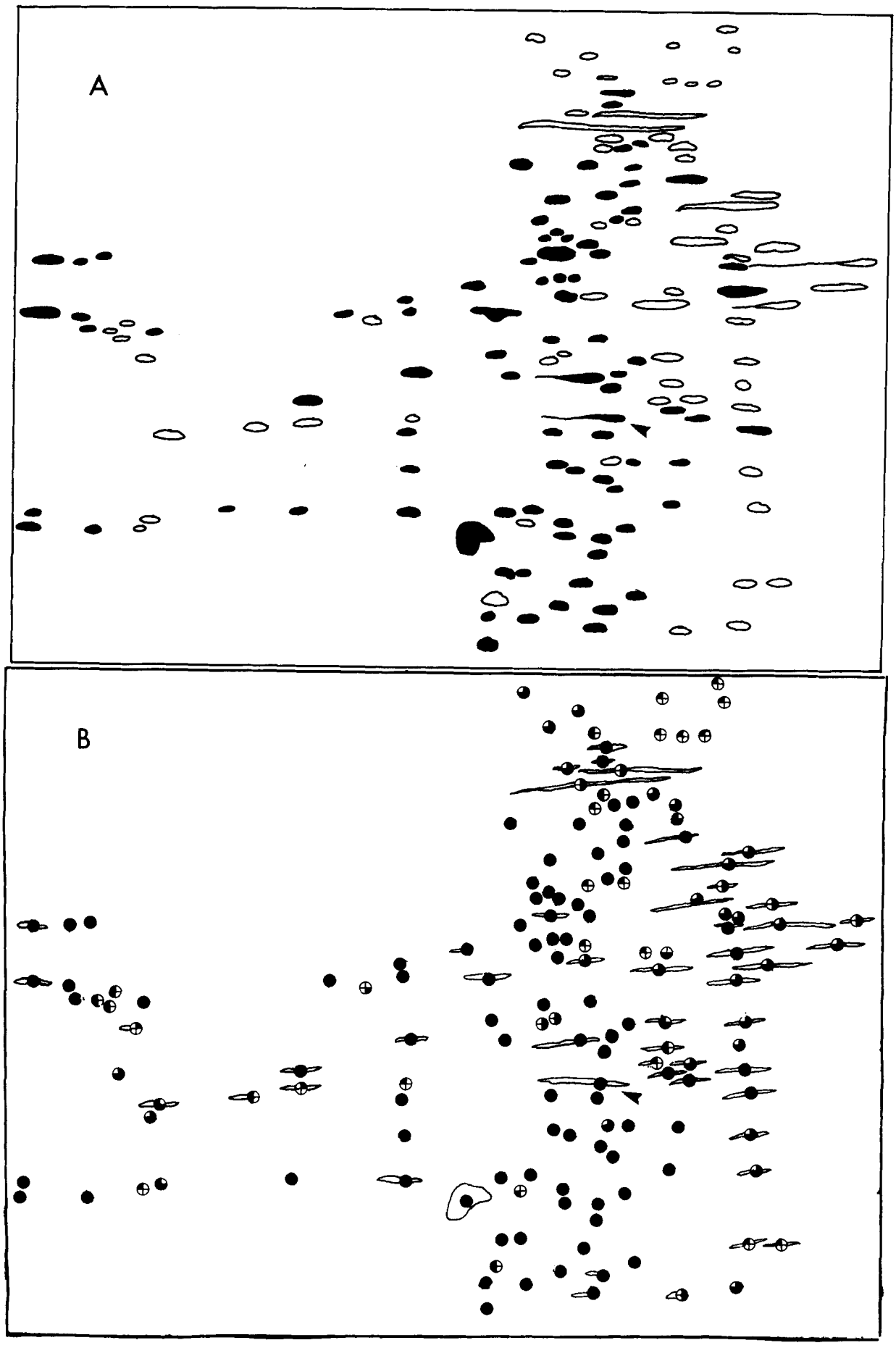
External Proteins

Cell surface-specific radioiodination was used to identify the external cell proteins and to study their developmental changes. The reagent Iodo-Gen has been used by Markwell and Fox (17) to achieve selective labeling of the external proteins of Sindbis and Newcastle Disease viruses which, as they point out, are relatively fragile systems.

Control experiments were performed to establish the surface specificity of labeling with the reagent on *D. discoideum* cells. Cells at early and late interphase, aggregation, and tip formation were labeled, lysed, and the 5,000 *g* pellet was fractionated on one-dimensional gels. The gels were stained with Coomassie Blue, dried, and exposed to film. Fig. 6 compares the radioactivity and staining profiles of the gel. While many proteins are iodinated, actin and the other major Coomassie-stained bands are not detectably labeled. Since it is unlikely that all the major cellular proteins lack accessible tyrosyl groups, this result indicates surface-specific labeling. Crude iodinated membranes from cells at early and late interphase, aggregation and tip formation were fractionated on two-dimensional gels. Fig. 7, *a–d* shows the autoradiograms obtained from these gels. Despite the radiographic spreading of the iodine label, it can be seen that most of the major species are conserved through development. A major surface protein (at ~58,000 daltons) is found at all four stages, though it changes in relative intensity. A more notable change is the disappearance of the cluster of spots below and to the left of the major surface protein at the aggregation stage (shown by arrows). A new species, which appears at the aggregation stage, has a molecular weight of ~27,000, and displays heterogeneity in the electrofocussing dimension, is also identified with arrows. This species may be discoidin since a number of reports have identified cell surface discoidin, the molecular weight is consistent with published values (24, 25), and, in preliminary experiments, authentic discoidin (generously provided by Dr. Edward M. Berger, Worcester Foundation, Shrewsburg, MA) is found at this location (data not shown).

An interesting change is the transient appearance of an isoelectrically heterogeneous species of ~40,000 daltons at the aggregation stage and the parallel transient disappearance of a similarly heterogeneous species of slightly higher molecular weight (shown by arrows). It is possible that the species at aggregation are derived from the higher-molecular-weight forms by some reversible modification that decreases the apparent molecular weight. These and other minor changes can be better seen in the composites of Fig. 8, *a* and *b*. The same quadrant system of representing developmental changes as in Fig. 2 has been used.

A comparison of these surface proteins with the abundant plasma membrane proteins identified by uniform labeling is shown in Fig. 9. The membrane protein profile is derived from Fig. 4*a*. Many of the external species can be identified on this profile; they are shown as filled spots. Of the 52 spots on the iodination composite, 25 can be seen on the uniform labeling composite. These correspondences were arrived at by superimposition of the original gels. The first conclusion from


A
B

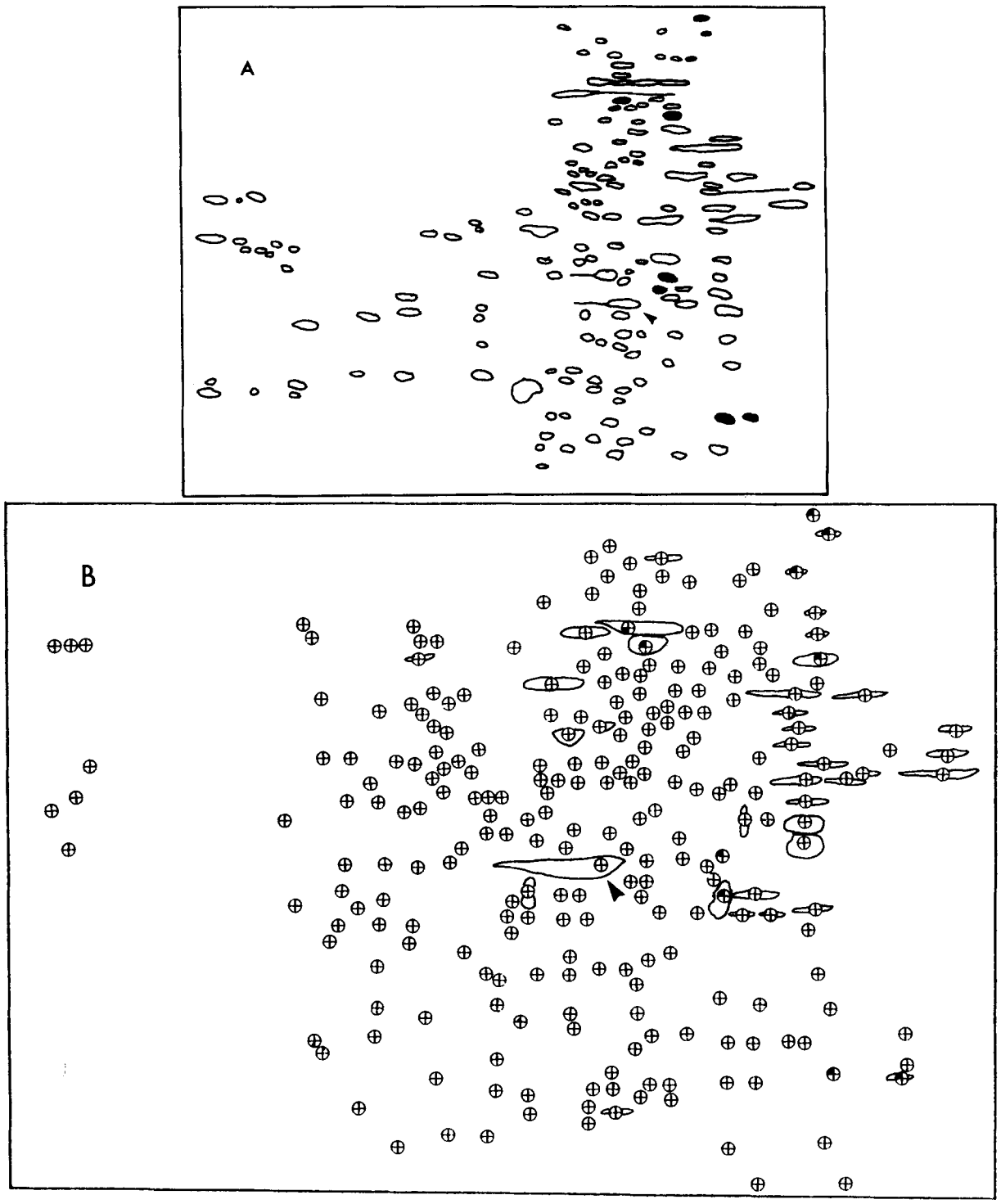


FIGURE 5 Comparison of uniform and pulse labeling of plasma membrane proteins. Composites derived from Figs. 4*A* and 2*B* are shown in this figure. New species that are observed to appear during uniform labeling at the various stages, which can be correlated as arising from increased synthesis as detected by pulse labeling, are indicated in this figure in the following manner. On the uniform labeling panel, namely *A*, these species are represented by filled spots. On the pulse-labeling panel, namely *B*, they are darkened in the appropriate quadrant as per the convention followed in Figs. 4*B* and 2*B*. Other spots or circles are left open.

FIGURE 4 Composites of uniformly labeled plasma membrane protein gels. The fluorograms of Fig. 3 were superimposed and compared. In *A*, all the spots in the four panels of Fig. 3 are shown so that the spot shapes approximate the originals. Species that are present in all the four fluorograms are indicated by filled spots. In *B*, each spot on the composite of *A* is represented by a circle. In some cases, characteristics of the original spots have been drawn around the circles to facilitate comparison. Each circle is divided into four quadrants. The quadrants that are filled indicate the particular stage at which the given species is detected, according to the following scheme: *Right upper*, early interphase; *right lower*, late interphase; *left lower*, aggregation; *left upper*, tip formation.

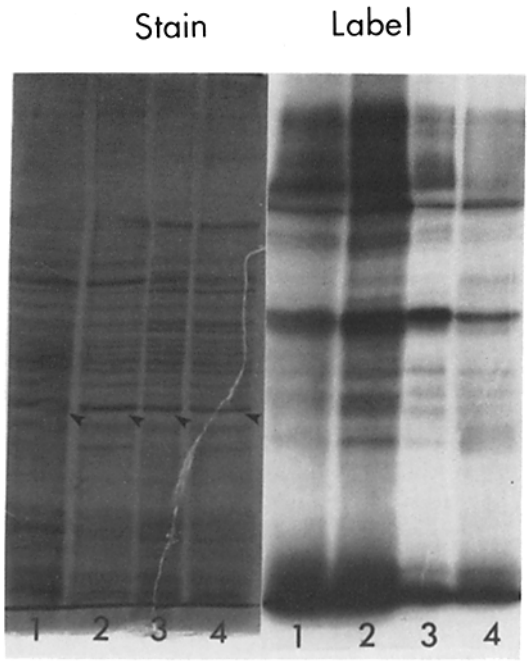


FIGURE 6 Surface specificity of Iodo-Gen labeling. Cells at four stages of development were harvested and labeled with Iodo-Gen as described in Materials and Methods. The labeled cells were lysed and centrifuged at 5,000 *g* for 20 min, and the crude membrane pellet obtained was solubilized and electrophoresed on one-dimensional gels. The pattern of stained bands is shown under *stain*. The pattern of labeled bands detected by autoradiography is shown under *Label*. Lane *1*, early interphase; lane *2*, late interphase; lane *3*, aggregation; lane *4*, tip formation. Each lane contained 100,000 cpm of protein-bound ^{125}I. The gel was exposed for 5 d. The position of the band corresponding to actin is marked by an arrow in each lane under *Stain*.

these comparisons is that, with one notable exception, the external proteins are minor components of the plasma membrane. The exception is the major surface protein which is also detected as a major membrane species in the uniform methionine labeling composite. This protein is synthesized only during the early and late interphase stages (Fig. 1) but persists as a major surface species through the tip formation stage.

Comparisons of the surface proteins with the newly synthesized plasma membrane proteins as detected by pulse labeling with [^{35}S]methionine (Fig. 1) were also attempted. In this case, however, unequivocal identification of surface species on the pulse labeling composite could not be made.

Whole Cell Proteins

Alton and Lodish (2) reported that by [^{35}S]methionine pulse labeling of whole *D. discoideum* cells, ~400 polypeptides could be detected during the total 24-h course of development and that, of these, ~100 changed in relative rate of synthesis, i.e., 25%. Steady-state levels were not determined. We ex-

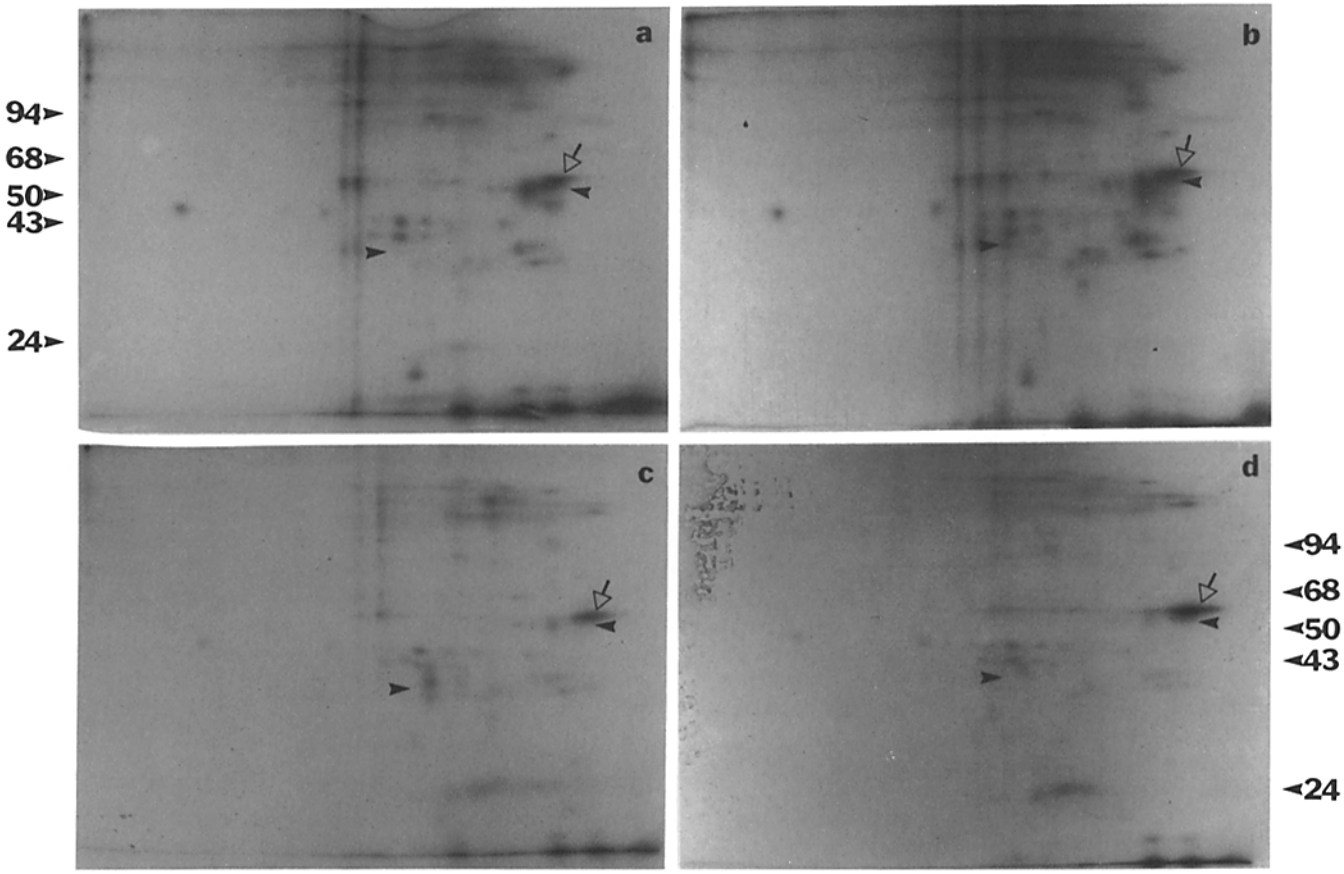
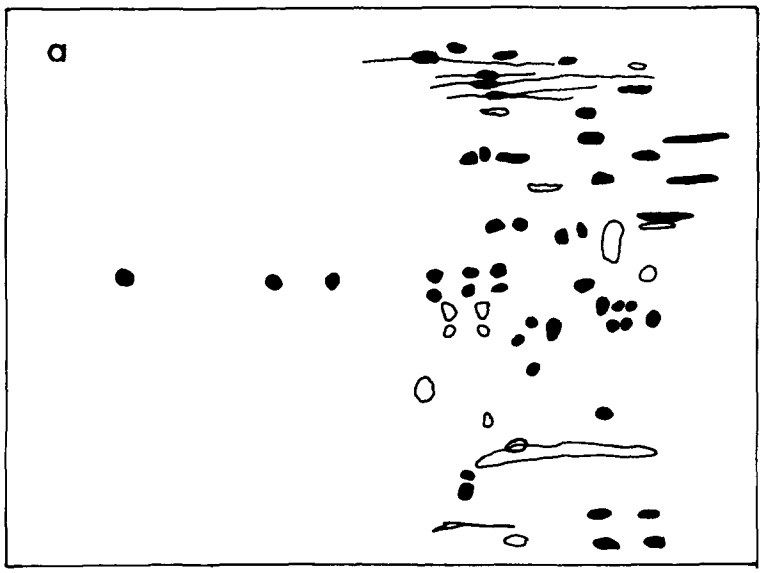


FIGURE 7 Two-dimensional gels of surface proteins. Cells at four stages of development were labeled with ^{125}I using Iodo-Gen as described in Materials and Methods. Crude membranes isolated from these cells by lysis and centrifugation at 5,000 *g* were electrophoresed on two-dimensional gels as described. Each gel was loaded with 200,000 cpm of protein-bound ^{125}I and was exposed for 20 d. Electrophoresis conditions were as in Fig. 1. The major external protein is identified by the open arrow.



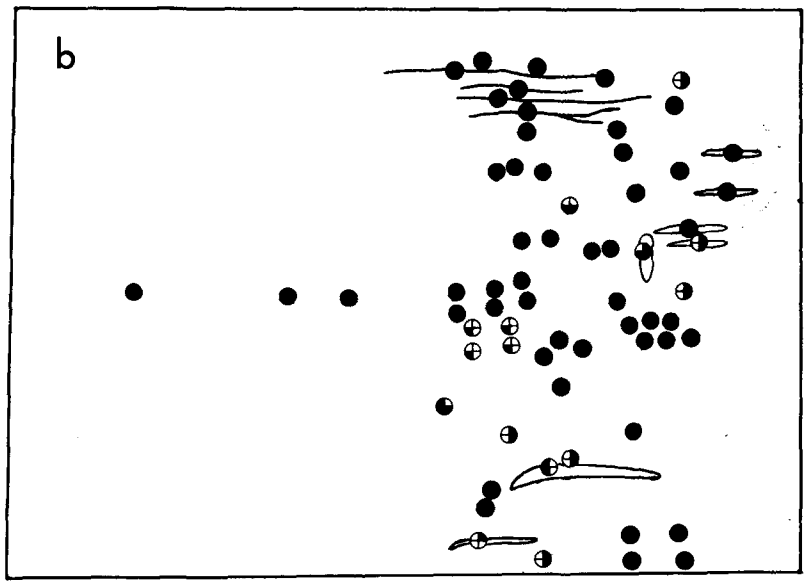


FIGURE 8 Composites of surface protein gels. The two-dimensional profiles of Fig. 7 were compared by superimposition, and composites that show all the spots detected on these gels were constructed. *a* shows a composite that has been drawn so that the spot shapes approximate the original. Species that are present at all the four stages of development considered are depicted by filled spots. *b* shows a schematic composite in which each spot is represented by a circle; in some cases, approximate spot shapes have been drawn around the circles to facilitate comparison. Each spot is divided into four quadrants that are darkened to indicate the developmental stage at which the given species is present according to the schedule below: *Right upper*, early interphase; *right lower*, late interphase; *left lower*, aggregation; *left upper*, tip formation.

amined whole cell proteins for several reasons. The first was to determine whether the steady-state pool of proteins would show fewer developmental changes than the newly synthesized proteins as was observed with plasma membrane proteins. The second reason was to assure that the difference between the reported 25% change and the extent of developmental regulation of plasma membrane proteins indicated by the preceding data was not due to differences in conditions. The third reason was to determine whether any plasma membrane proteins were of sufficiently high abundance to be detected in whole cell samples.

Fluorograms of gels of whole cell proteins after pulse-labeling for 1 h with [^{35}S]methionine are shown in Fig. 10. Developmental stages represented are early interphase, aggregation, and tip construction (Fig. 10, *a–c*, respectively). In Fig. 10*a*, 359 proteins can be identified and, of these, 329 are in common with aggregating amoebae (Fig. 10*b*). Aggregating amoebae show 20 new spots. Further data reduction by the methods described for pulse-labeled membrane proteins generates the data in Table III. The percentage change (27%) is similar to that reported by Alton and Lodish (2). Steady-state levels of whole cell proteins were analyzed by starvation after two generations of vegetative labeling with [^{35}S]methionine. The fluorograms are shown in Fig. 11, and the data are summarized in Table IV. The change over the 14-h period of development (20%) is less than that for newly synthesized species though the magnitude of the difference is considerably less than the plasma membrane case. The analysis has not been done as thoroughly as for plasma membranes, but these data also appear to be internally consistent. For example, two proteins (in boxes in Figs. 10 and 11) are detected at late developmental stages by uniform labeling and are seen to be actively synthesized earlier by pulse labeling.

Major Plasma Membrane Proteins

When radioactive plasma membrane proteins and whole cell proteins are compared (Figs. 1 and 10 or 3 and 11), the

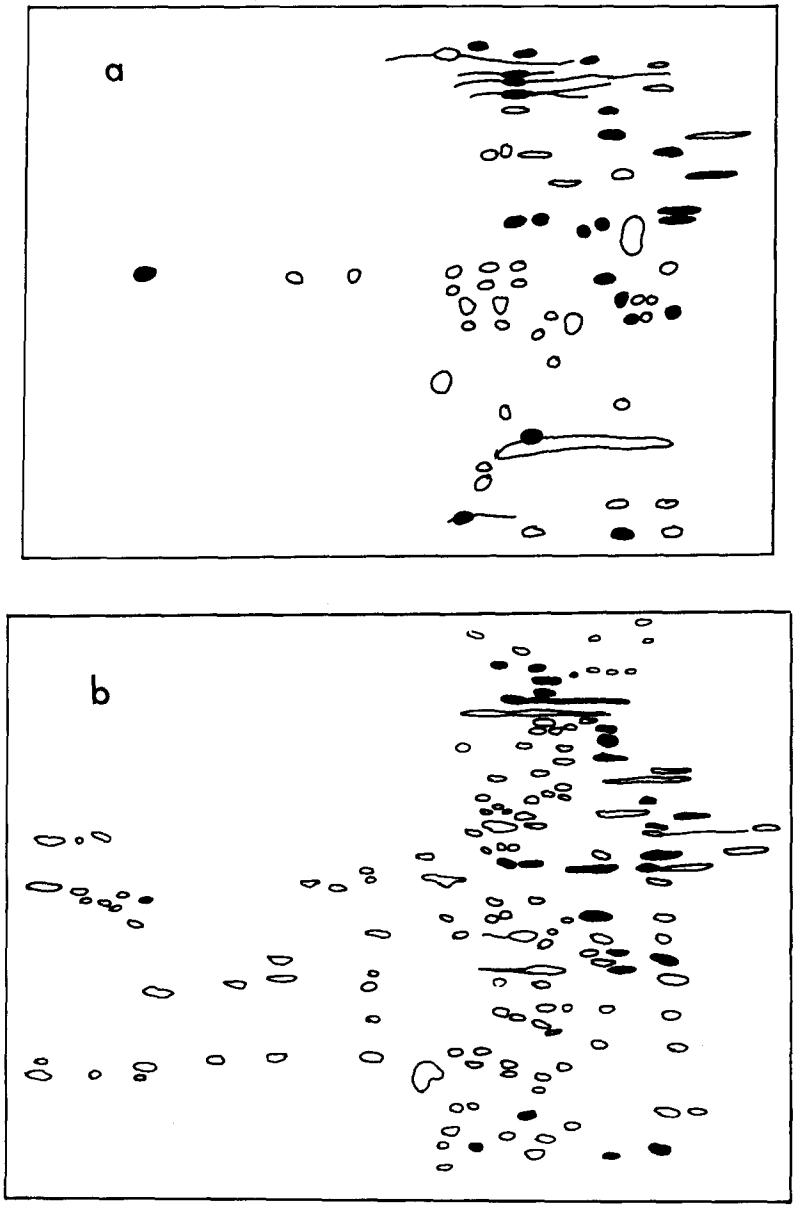


FIGURE 9 Comparison of uniformly labeled proteins with external proteins. Composites derived from Figs. 8a and 4A are presented in this figure. Filled spots in the panels indicate spots detected both by surface iodination (a) and by uniform labeling (b).

profiles are remarkably different. Most plasma membrane proteins are minor constituents of the cell. A few plasma membrane proteins are present in such large amounts that they may be detected in whole cell preparations. Examples are actin and the doublet of spots above and very slightly to the left of actin. Some species in the acidic regions of the whole cell gels can also be seen in plasma membranes.

DISCUSSION

One of the most significant conclusions that can be reached from these studies is that there are two general classes of plasma membrane proteins. The first is composed of high-abundance species that are largely conserved through development and presumably reflect functions common to all stages of the life cycle, i.e., "housekeeping species." The second is a class of low-abundance proteins that show drastic developmental changes and often very precise stage specificity. These presumably serve stage-specific functions that are critical for development. The existence of two classes shown here has been implied by previous work from other laboratories. Coomassie Blue or amido black staining of one- and two-dimensional gels has detected relatively little developmental regulation whereas more developmental change has been reported when pulse metabolic labeling has been used (4, 8, 9, 11, 13).

Ono et al. (13) reported that the profile of steady-state species as determined by Coomassie Blue staining was nearly completely distinct from the profile of newly synthesized proteins, consistent with the results reported here. However, since many plasma membrane proteins are glycoproteins and since Coomassie Blue does not stain most glycoproteins, we made comparisons based on methionine content. By this criterion, most stage-specific proteins do not accumulate to levels detectable by long-term labeling, and this is compatible with observations that several known developmentally regulated plasma membrane proteins are expressed at the cell surface in a limited number of copies per cell. Examples are the cell surface receptors for cAMP (26–28), the membrane-bound cAMP phosphodiesterase (29), contact sites A (30, 31), a cell surface glycoprotein (7), and discoidin (25, 32).

The most dramatic changes in plasma membrane proteins

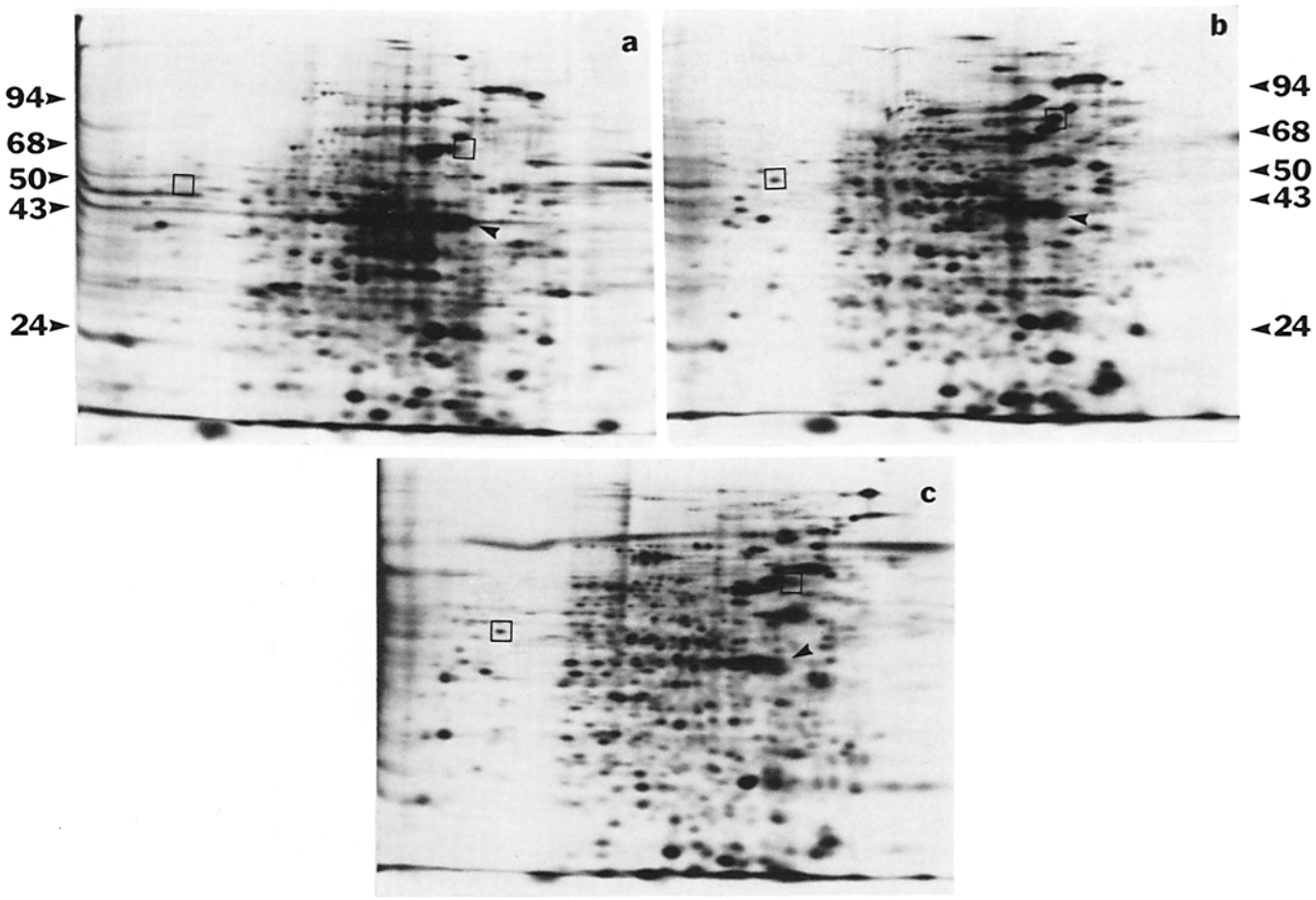


FIGURE 10 Fluorograms of pulse-labeled whole cell proteins. Cells at three stages of development were pulse labeled with [^{35}S]methionine and electrophoresed on two-dimensional gels as described in Materials and Methods. (a–c) Fluorograms obtained from early interphase, aggregation, and tip formation stages, respectively. Each gel was loaded with 500,000 cpm of protein-bound ^{35}S, and exposures were for 20 d in all three cases. Other details are as in Fig. 1.

TABLE III

Developmental Changes of Whole Cell Proteins Detected by Pulse Labeling

Stages compared	Total proteins scored	Common proteins	Percentage change
Early interphase—aggregation	379	329	13
Aggregation—tip formation	491	406	17
Early interphase—tip formation	269	197	27

Pairs of fluorograms of whole cell proteins from pulse-labeled cells (Fig. 10) were compared by superimposing and visual scoring of changes. The data under "Total proteins scored" do not represent all the species that are seen in Fig. 10; the only ones scored were those for which a clear positional commonality or lack thereof could be established, since many of the regions of the fluorograms are very crowded.

previously reported were those of Ono et al. (13). Those authors showed that on two-dimensional gels 90 of 200 plasma membrane proteins varied in level over the period through aggregation, ~60% of which showed apparent on/off stage specificity. This would, by our scoring, translate into ~30% rather than 86% change. However, the pulse labeling used by Ono et al. did not include the tip formation stage at which we observed the most drastic changes. Other probable bases for the difference are that the data shown here were obtained with 25–30-fold rather than 8–10-fold purified membranes and 1-h rather than 2-h pulse labels. Detection of the extensive developmental regulation of newly synthesized proteins requires two-dimensional electrophoretic analysis as also reported by Ono et al. (13). When the same samples are analyzed on one-dimensional gels, the observed changes are substantially reduced due to co- or overlapping migration of multiple species.

Two general conclusions can be derived from the results of the radioiodination studies. The first is that, with one exception, the external proteins are minor components of the plasma membrane. Secondly, even in this minor pool, developmental changes are relatively few; many of the species that are critical for development are presumably present in very low amounts, below our detection limits. This is consistent with the results of the methionine-labeling studies. As an example, one of the first developmentally regulated surface species to be identified was contact site A, a glycoprotein of molecular weight 80,000 (30, 31). We have not been able by iodination to detect a new surface species appearing at this molecular weight on two-dimensional gels during the course of development though methionine pulse-labeling does detect candidates. Lam and Siu (12) have reported iodination of glycoprotein 80 as observed on a one-dimensional gel. However, on two-dimensional gels, this phosphoprotein produces a number of stuttered, isoelectrically heterogeneous spots (33–

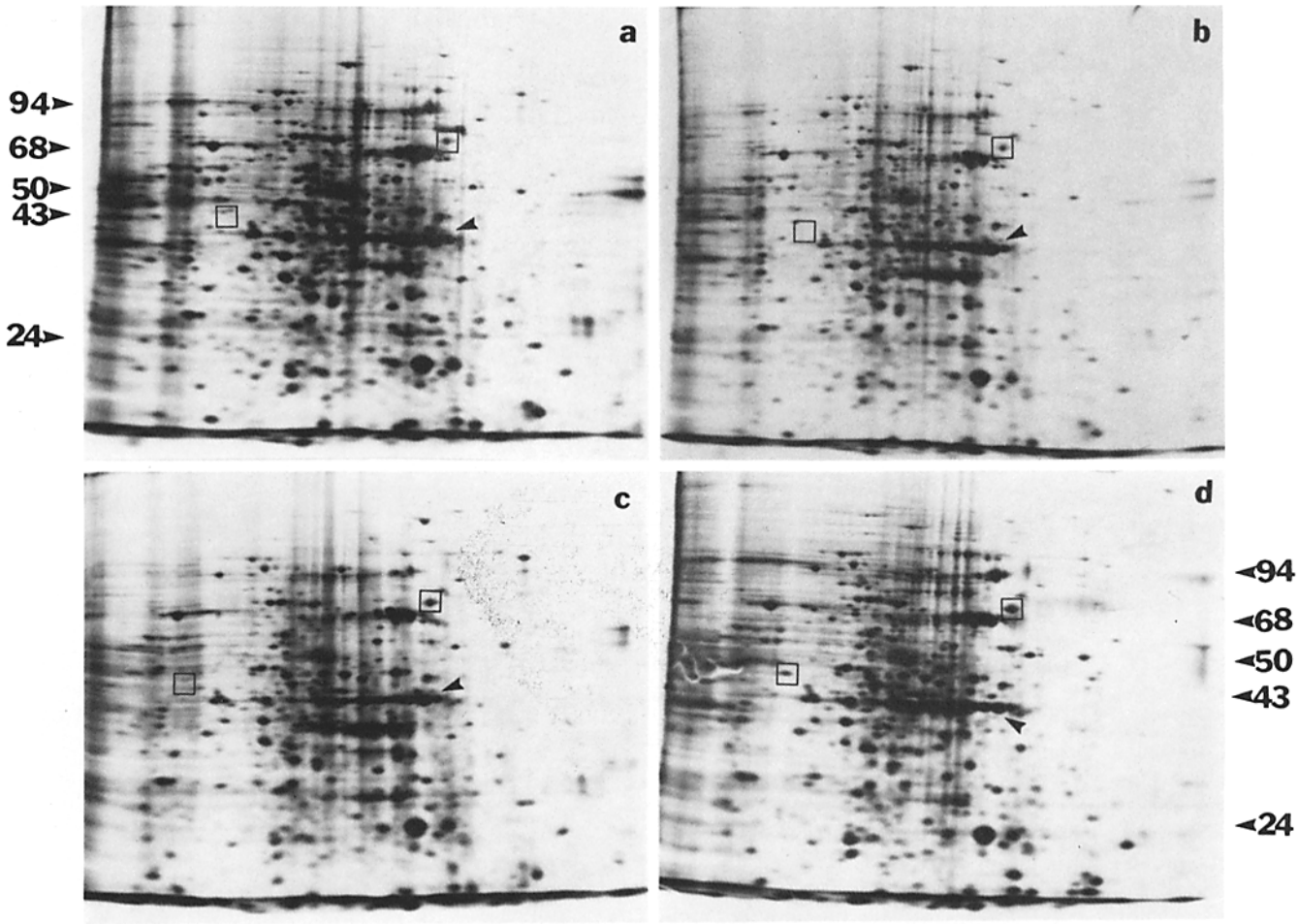


FIGURE 11 Fluorograms of uniformly labeled whole cell proteins. Cells were grown for two generations in [^{35}S]methionine-containing medium, and development was initiated. At the appropriate stages, amoebae were harvested and fractionated on two-dimensional gels. (*a–d*) Fluorograms of gels corresponding to early and late interphase, aggregation, and tip formation stages, respectively. Each gel was loaded with 500,000 cpm of protein-bound ^{35}S and was exposed for 20 d. Other details are as in Fig. 1.

TABLE IV

Developmental Changes in Whole Cell Proteins Detected by Uniform Labeling

Stages Compared	Total proteins scored	Common proteins	Percentage change
Early—late interphase	523	512	2
Late interphase—aggregation	538	499	7
Aggregation—tip formation	539	477	12
Early interphase—tip formation	553	441	20

Pairs of fluorograms from uniformly labeled whole cells (Fig. 7) were compared by superimposing and visual scoring of changes. Only spots that either disappeared or appeared were scored as changes; spots that changed only in intensities were not scored.

37). Since contact site A comprises only 1% of the cell surface antigens (30) and disperses into a number of spots, it may simply be below our detection limits for iodination.

Another developmentally regulated protein that has been detected on the cell surface is the lectin discoidin which has been postulated to be involved in cellular cohesion (25, 32). As mentioned in the Results, a candidate for discoidin can be identified by radioiodination. This species cannot, however, be seen on the methionine-labeling gels, probably because this protein has 11 tyrosine residues and only one methionine per polypeptide chain (38). Also, we, as well as Alton and Lodish (2), have worked with axenic strain A3 which is known to precociously acquire a few early developmental components during growth (32, 39), including discoidin. The presence of this species in this strain at the earliest times studied (Fig. 7) is, therefore, compatible with its identification as discoidin.

There were two major changes observed by iodination. Disappearance of the cluster of spots around the major cell surface protein has not been reported by others. However, since the spots are very close to the major external species, this change is not detected on one-dimensional gels (compare Figs. 6 and 7). The transient appearance of the 38,000-dalton protein has previously been observed by Hoffman and McMahon (8) using pronase treatment of cells to identify surface species.

Toda et al. (10) have used periodate oxidation with [^{3}H]-borohydride reduction to label cell surface glycoproteins and have analyzed them on one- and two-dimensional gels. The two-dimensional pattern of external glycoproteins detected is qualitatively similar to the ones of Fig. 7, though exact comparisons cannot be made because of differences in labeling and electrophoretic conditions. These authors report extensive developmental alteration (45 out of 63 spots). Since their

evaluation is based on relative spot intensities and ours is based on apparent on/off differences, the results are not directly comparable.

The experiments described have been directed at two aspects of developmental regulation of the plasma membrane proteins: the amount of each species present at each stage and the synthesis of new proteins at each stage. A third way in which the cell can exert developmental control over its plasma membrane proteins, i.e., selective degradation, has not been addressed in these studies. However, from the results described, it can be inferred that mechanisms exist that are selective for the removal of specific membrane proteins. For instance, the major surface protein that is synthesized exclusively at the early and late interphase stages (Fig. 2) persists through the tip formation stage while other surface species (Figs. 7 and 8) disappear during the same interval.

The profiles of plasma membrane and total cellular proteins on two-dimensional gels are almost totally distinct. A few plasma membrane proteins are of such high abundance that they can be detected in the whole cell profile. Actin is in this category, but the cellular function of the other high-abundance species is currently unknown. Since the plasma membrane and total cellular profiles are so distinct, the changes in one pool can effectively be analyzed without correction for contributions by the other. When these comparisons are made, both pulse- and long-term labeling show greater changes (86 and 33%) in the plasma membranes than in whole cells (27 and 20%). This is consistent with the expectation that the plasma membrane, being the interface between cells and their environment, is more intimately involved in the processes of cellular differentiation than the internal domains of the cells.

The two classes of plasma membrane proteins are not totally distinct. For example, some of the new plasma membrane species observed by long-term labeling can be identified as newly synthesized at the preceding stage in the pulse-labeling profiles. However, there are some species that appear to increase in levels during development and for which de novo synthesis has not been detected. Since we have only considered four 1-h intervals of the development program, one possibility is that the synthesis of these particular proteins may have been overlooked in a system under finely timed controls. At the same time, the preceding discussion has assumed that the observed changes were due to altered polypeptide synthesis, and this need not be the case for all species. Coffman et al. (33) have reported developmental changes in phosphorylation of plasma membrane proteins, and we (40) have previously reported that the processing at *N*-linked oligosaccharides undergoes a sharp transition at the tip formation stages. In addition to these posttranslational modifications, another possibility is the binding of pre-existing proteins to newly synthesized membrane receptor sites. These phenomena may account for some of the changes observed. However, the great majority of the changes observed in the gel profiles are not suggestive of simple shifts in mobility and probably reflect regulation at the level of gene expression.

It is of note that all experiments to test steady-state levels of proteins, by long-term metabolic labeling, surface iodination or Coomassie Blue staining, have been highly reproducible. Both the pattern of spots and the percentage changes have been virtually identical. For the pulse-labeling experiments, while the percentage changes are essentially the same from experiment to experiment, the two-dimensional protein profiles are not exactly reproduced. This is presumably because the developmental changes in the newly synthesized plasma membrane proteins are so extensive and stage specific, and because the exact time at which a given developmental stage is reached can vary slightly between experiments.

Estimates of the number of genes utilized for *D. discoideum* development have ranged from several hundred (41) to several thousand (42). Recently, Blumberg and Lodish (43) have identified ~3,000 new transcripts that appear at the stage of tight cell-cell contact and tip formation. This is also the stage at which Alton and Lodish reported the major changes in protein synthesis in whole cells and showed these changes to be dependent on cell-cell contacts (2, 44). They observed relatively fewer changes in early development, and this is consistent with estimates of the number of genes required for aggregation, which range from 50 (45) to 126 (41). Indeed, one might expect the major cellular transitions to occur on differentiation of aggregated amoeboid cells into prestalk and prespore cells.

We also observe the major changes in plasma membrane proteins at this stage but observe as well large numbers of changes during early interphase and aggregation. Most of these changes could not be detected in whole cell preparations and would not have been seen by Alton and Lodish (2). We speculate that while smaller numbers of changes in gene expression are required for aggregation, a high proportion of these may be in plasma membrane proteins required for the transition from independent cellular behavior during growth to the social chemosensory and cohesive behavior observed during aggregation.

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