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Hormonal Control of Mitotic Development in Tobacco Protoplasts

TWO-DIMENSIONAL DISTRIBUTION OF NEWLY-SYNTHESIZED PROTEINS

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YVES MEYER AND YVETTE CHARTIER
Laboratoire de Physiologie Végétale, Université de Perpignan, Avenue de Villeneuve, F-66025 Perpignan-Cedex, France

ABSTRACT

Two-dimensional separation of proteins newly synthesized by tobacco mesophyll protoplasts cultivated *in vitro* allows us to detect, reproducibly, 257 spots. The pattern is extremely stable throughout the three days of culture, the intensity of only 24 spots varying during this time. The absence of cytokinin (N^6-benzyladenine) in the culture medium prohibits entry into S phase but does not modify the pattern, indicating that none of the observed proteins is specifically synthesized in S, G_2, or M phases. The presence of 2,4-dichlorophenoxyacetic acid is necessary for the mitotic development of protoplasts. It induces the appearance of one protein, increases the level of another, and reduces that of eight others. All proteins sensitive to auxin belong to the group of proteins the levels of which vary during culture.

The isolation of mesophyll protoplasts from their tissue of origin frees them from the influences which maintained the cells in a differentiated state. Protoplasts in culture do not retain the characteristics of the cells from which they originate: they lose photosynthetic activity, but regain mitotic activity and synthesize a cell wall. Although the disappearance of photosynthetic activity occurs regardless of the culture medium, both the reformation of the cell wall and the entry into mitosis do take place in the presence of auxin and cytokinin. We have previously shown (4, 14) that if auxin is absent from the medium, mitotic development does not begin, whereas in the absence of cytokinin this development starts but later stops just before S-phase. The study of this experimental system is thus likely to provide information on the modes of action of auxin and cytokinin, as well as on the development of the mitotic program in a plant cell.

The results presented here concern the study of newly synthesized proteins in protoplasts cultivated in complete medium or in medium deficient in auxin or cytokinin. To obtain the best resolution currently available, we compared the patterns obtained by two-dimensional separation of proteins: electrofocusing followed by electrophoresis under denaturing conditions (16, 18).

MATERIALS AND METHODS

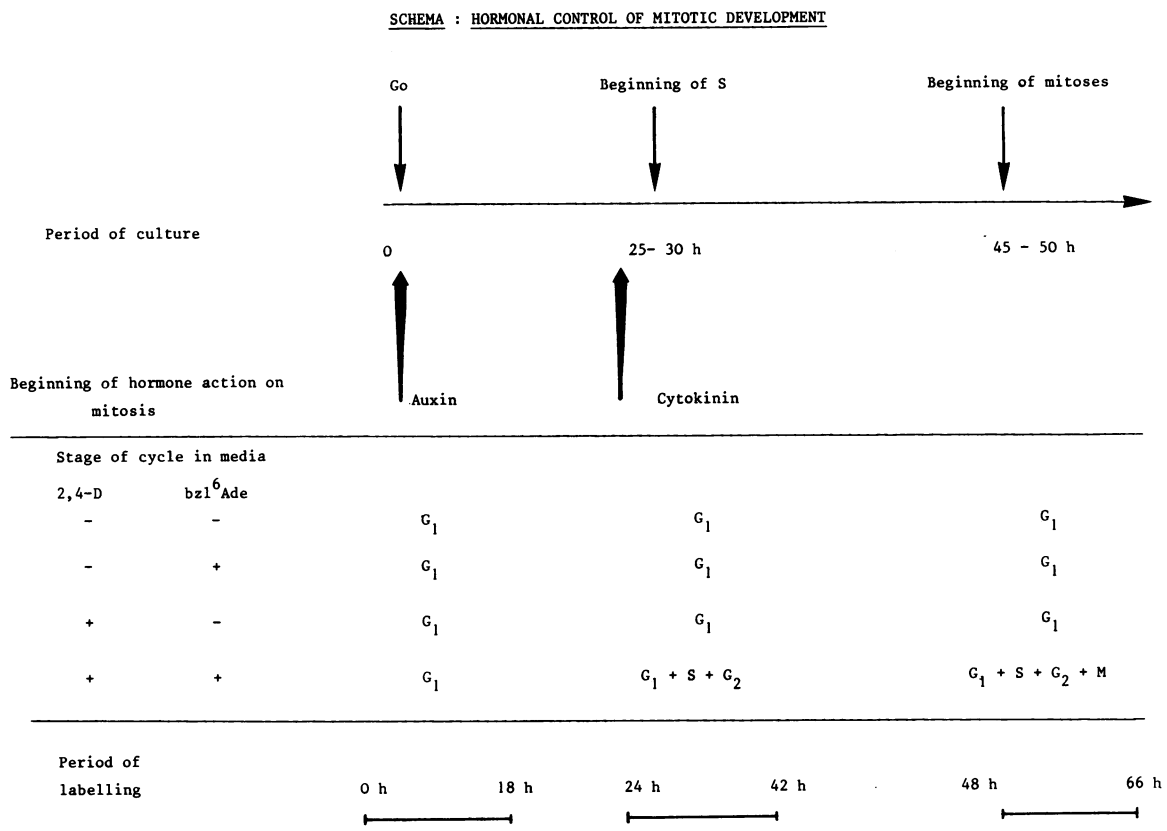
Preparation and Culture of Protoplasts. Tobacco mesophyll protoplasts (*Nicotiana tabacum* var. Maryland) were prepared and cultivated as described previously in medium WO.6 (12, 13) in the absence or presence of 1 mg/l of auxin 2,4-D or cytokinin N^6-benzyladenine.

Radioactive Labeling of Cells. Protoplasts were labeled by adding 33 μCi [^{35}S]methionine (575 Ci/nmol, Commissariat à l'Energie Atomique) to 2.5 ml culture containing 3 to 10 × 10^4 protoplasts for the following periods: 0 to 18 h, 24 to 42 h, 48 to 66 h. These periods were chosen on the basis of our knowledge of mitotic development in cultivated protoplasts (4, 14): in complete medium the 0 to 18 h labeling was carried out on cells which are all in G_1; the entry into S phase begins asynchronously after about 25 to 30 h of culture; the 24 to 42 h labeled cultures consist of a mixture of cells in G_1 and S phases, with a few cells in G_2; the first mitoses appear after about 45 to 50 h of culture, while the 48 to 66 h labeling is carried out on a population composed of cells at all stages of the cell cycle. Cells cultivated in the absence of one or the other hormone remain blocked in G_1 and serve as controls of a nonmitotic development (as summarized in the schema).

Preparation of Samples. After labeling, protoplasts from one culture dish were collected by centrifugation. Total protein was obtained by lysis of protoplast pellets in 100 μl of a solution containing 9.5 M urea, 2% LKB ampholines (pH 3–10), 5% β-mercaptoethanol, and 0.2% SDS. This extract can be frozen and stored at −70°C. Just before electrofocusing, the extract was mixed with two volumes of a solution containing 9.5 M urea, 2% Nonidet P 40, 2% LKB ampholines (pH 3.5–10), and 5% β-mercaptoethanol and centrifuged 10 min in Eppendorf tubes. In some experiments "buffer-soluble" proteins were obtained by vortexing protoplast pellets for 30 s in 50 μl of 100 mM Tris-HCl, 10 mM EDTA (pH 8.0). To the supernatant recovered after centrifugation (15 min, 10,000*g*) was added urea to 9.5 M, Nonidet P 40 to 2%, LKB ampholines (pH 3.5–10) to 2%, and β-mercaptoethanol to 5%.

Electrofocusing. Twenty μl of the resulting supernatant were loaded onto cylindrical, nonequilibrium, electrofocusing gels (length 12 cm, diameter 2 mm) containing 4% acrylamide, 9 M urea, 2% Nonidet P 40, and 2% ampholines (pH 3.5–10). Samples were loaded on the acid end of the gel (17) and covered with 20 μl overlay solution (8 M urea, 1% ampholines, 2% Nonidet P 40, [16]). Electrofocusing was carried out for 30 min at 100 v, 60 min at 200 v, 30 min at 300 v, 60 min at 400 v, and 120 min at 500 v (a total of 1800 v h). Gels were subsequently equilibrated in 10 ml buffer O (10% glycerol, 5% β-mercaptoethanol, 2.5% SDS, 62.5 mM Tris-HCl [pH 6.8]) containing 0.01% bromophenol blue for 30 min then frozen and stored at −70°C.

Denaturing Electrophoresis. After thawing, first dimension cylindrical gels were dialyzed a further 30 min in 10 ml fresh buffer O containing 0.01% bromophenol blue. The second dimension gels were prepared in a Bio-Rad apparatus (Model 220) using 1.5-mm spacers. The separating gel was 12.5% acrylamide, 0.1% SDS, 0.37 M Tris-HCl (pH 8.8), and the stacking gel 0.125 M Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 0.1% SDS, 5% glycerol, 1% Pharmacia IEF agarose. Other tested kinds of agarose are totally unsuitable. The stacking gel was poured at about 50°C and the



first dimension gel inserted directly on top.

Migration was carried out at 20 mamp/gel until the bromophenol blue reached the lower buffer (about 4–5 h). The following mol wt markers were used: lysozyme (mol wt 14,300), carbonic anhydrase (mol wt 30,000), ovalbumin (mol wt 46,000) BSA (mol wt 69,000), phosphorylase b (mol wt 92,500), myosin (mol wt 200,000) as ^{14}C methylated form (Amersham). The gels were subsequently dehydrated 2 h in three changes of dimethylsulfoxide, impregnated 2 h with 5% PPO in dimethylsulfoxide, rehydrated 1 h in five changes of distilled H_2O and then dried under vacuum on a gel drier. Fluorographs were obtained by exposure at −70°C to Kodak Royal X-Omat film (2, 10).

RESULTS

Two-Dimensional Separation of Plant Proteins. Although the method of O'Farrell *et al.* (16, 17) is routinely used in the analysis of bacterial or animal extracts, it has seldom been used for plant material and, in all cases, the resolution is inferior to that obtained with the former systems. Our results do not escape this problem, as we detect only about 250 proteins, compared with 500 to 600 for animal or bacterial extracts on similar-sized gels. This is partly due to the low protein content of plant extracts, which leads to less efficient (or more difficult) radioactive labeling, and above all, to the presence of substances which interfere with electrofocusing. For example, if protoplast extracts are loaded onto the basic end of the gel, as is usual in equilibrium focusing, very few proteins enter the gel. Furthermore, these proteins are not focused but form streaks, probably due to the presence of positively charged substances which tend to migrate through the gel but which block it.

The procedure which we use at present consists in loading samples at the acid end of first dimension gels and using agarose and β-mercaptoethanol in the stacking gel. It allows the characterization of about 250 polypeptides from protoplast extracts. Although this resolution is not comparable with that obtained with animal extracts, it compares favorably with the best published results on the separation of plant proteins (11, 15, 20).

Figure 1 shows that three or four fluorographs, representing different times of exposure of one gel, are necessary to exploit the whole gel. Most major proteins are neutral at the time of culture (Fig. 1A). Later in culture, several basic proteins become major (Fig. 2). The detection of less radioactive proteins requires longer exposure times (Fig. 1, B and C). Those present in the neutral zone are masked by diffusion of the major proteins, but proteins in the acid (right hand side of the gel) and basic (left hand side) zones are well resolved.

Fractionation of samples into buffer-soluble and insoluble proteins allows us greatly to improve two-dimensional separation of buffer-soluble proteins (Fig. 3, E–H). However, we have encountered difficulty in obtaining perfectly reproducible solubilization and, for this reason, have only analyzed buffer-soluble proteins in regions where the resolution of total protein is weak. We have analyzed at least three samples solubilized independently for each treatment.

Modification of the Protein Map during Culture in Complete Medium. Figure 2 shows the gels obtained from extracts of protoplasts labeled after different times of culture. The general distribution is unchanged although considerable variation in the levels of 24 spots are noted. The radioactivity of 22 of these increases considerably (black arrows), while the radioactivity of two spots decreases (white arrows).

Effect of Hormones.

Lack of Effects of Cytokinin. No effect of cytokinin on the protein pattern was detected. For example, the gel shown in Figure 3B (medium with auxin but lacking cytokinin) shows no differences from that in Figure 2 B′ (complete medium).

Effect of Auxin. In contrast to the results described above, the

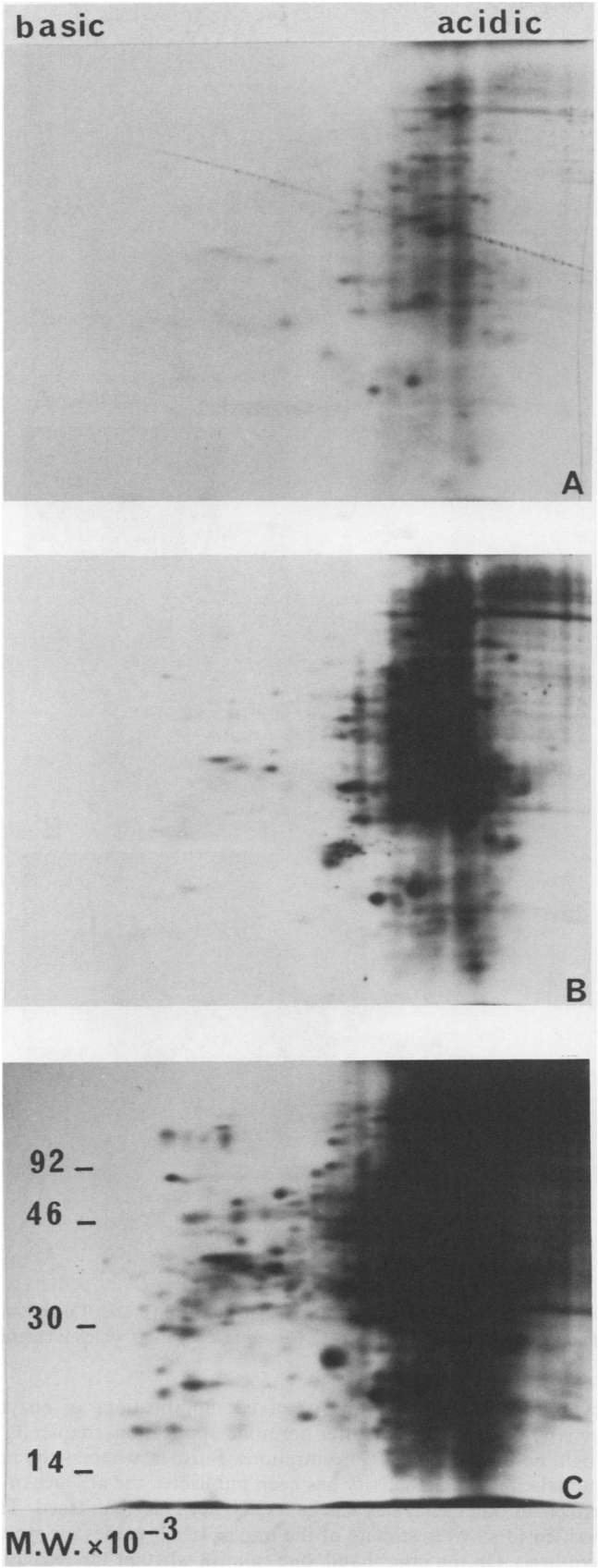


FIG. 1. Pattern of protein newly synthesized by protoplasts in culture. Protoplasts were cultivated in medium lacking 2,4-D but containing N^6-benzyladenine, and labeled between 0 and 18 h. Fluorographs were obtained after exposures of 6 h (A), 4 days (B), or 7 days (C) at −70°C.

presence of auxin modifies the protein pattern. The most striking effect is the diminution in the radioactivity of certain spots in the presence of 2,4-D in the culture medium. During the first 18 h of culture, the basic protein, a, (mol wt 36,000 daltons) is less in the presence of auxin (Fig. 3, A and B). This effect is maintained throughout the culture period. New differences appear between 24 and 42 h of culture (Fig. 3, C and D), basic proteins a′ (mol wt 36,000 daltons) a_1 (mol wt 38,000 daltons) b, b′ (mol wt 26,000 daltons) and c (mol wt 19,000 daltons) appear, but are less in the presence of auxin. In the region indicated by the dotted lines (neutral pH, proteins of about 30,000 daltons) an increase in radioactivity effected by the presence of auxin is detectable, even though the resolution is poor in this zone with total protein. Analysis of buffer-soluble proteins gives much better resolution (Fig. 3, E–H). It can be seen that the situation in this zone is relatively complicated. Protein α is only detectable when auxin is present in the culture medium, protein β is increased in presence of auxin although it is detected also in absence of auxin, and proteins d and e are more abundant in the absence of the hormone.

Figure 4 is a summary showing the positions of 257 different newly synthesized protoplast proteins and their modification as a function of the length of the culture period and of the presence or absence of auxin. The information obtained by analysis of the buffer-soluble proteins in the zone contained within the dotted lines has been added to that obtained with total protein. With the exception of some spots which are undetectable in protoplasts cultivated between 0 and 18 h, and of α which is undetectable in the absence of auxin, all other spots were found in all culture media and at all times of labeling. In most cases, the identical nature of spots from different extracts was confirmed by comigration of different protein preparations in the same gel.

DISCUSSION

Protein Pattern and Mitotic Development. The absence of an effect of cytokinin on the protein pattern indicates that none of the proteins seen on the gel is specifically synthesized during S, G, or M phases, as these phases do not occur in the absence of cytokinin (schema). It should be noted that in synchronized animal cells (a much more favorable system for this type of study) the only differences observed were variations in the level of synthesis of a few proteins. No syntheses closely linked to the phase of the cell cycle were detected (3).

The existence of proteins functional at certain stages of the cycle is demonstrated by the existence of mitotic mutants. However, it is possible that the products of these genes are present in quantities undetectable by our methods, or that these proteins are synthesized throughout the cycle, although they are only active at a particular stage.

Protein Pattern and Differentiation. The most striking result of this work is the stability of the pattern of newly synthesized proteins at different stages of culture and in different media. This stability is particularly astonishing when one observes the spectacular cytological development undergone by protoplasts in culture (7).

In addition, we know that newly synthesized proteins in culture are very different from the proteins of mesophyll cells, as their patterns can be distinguished by monodimensional electrophoresis (5). The pattern observed is thus specific for the meristematic state.

While the majority of proteins remain unchanged throughout the culture period, the levels of several peptides vary, whereas others appear late in culture. It is interesting to note that all proteins sensitive to auxin belong to this latter category. It is possible that two types of protein exist for a given differentiation state: one group, the synthesis of which is invariable, characteristic of the differentiation state, and a second, strictly regulated group, sensitive to external effects (auxin, variation in the medium during

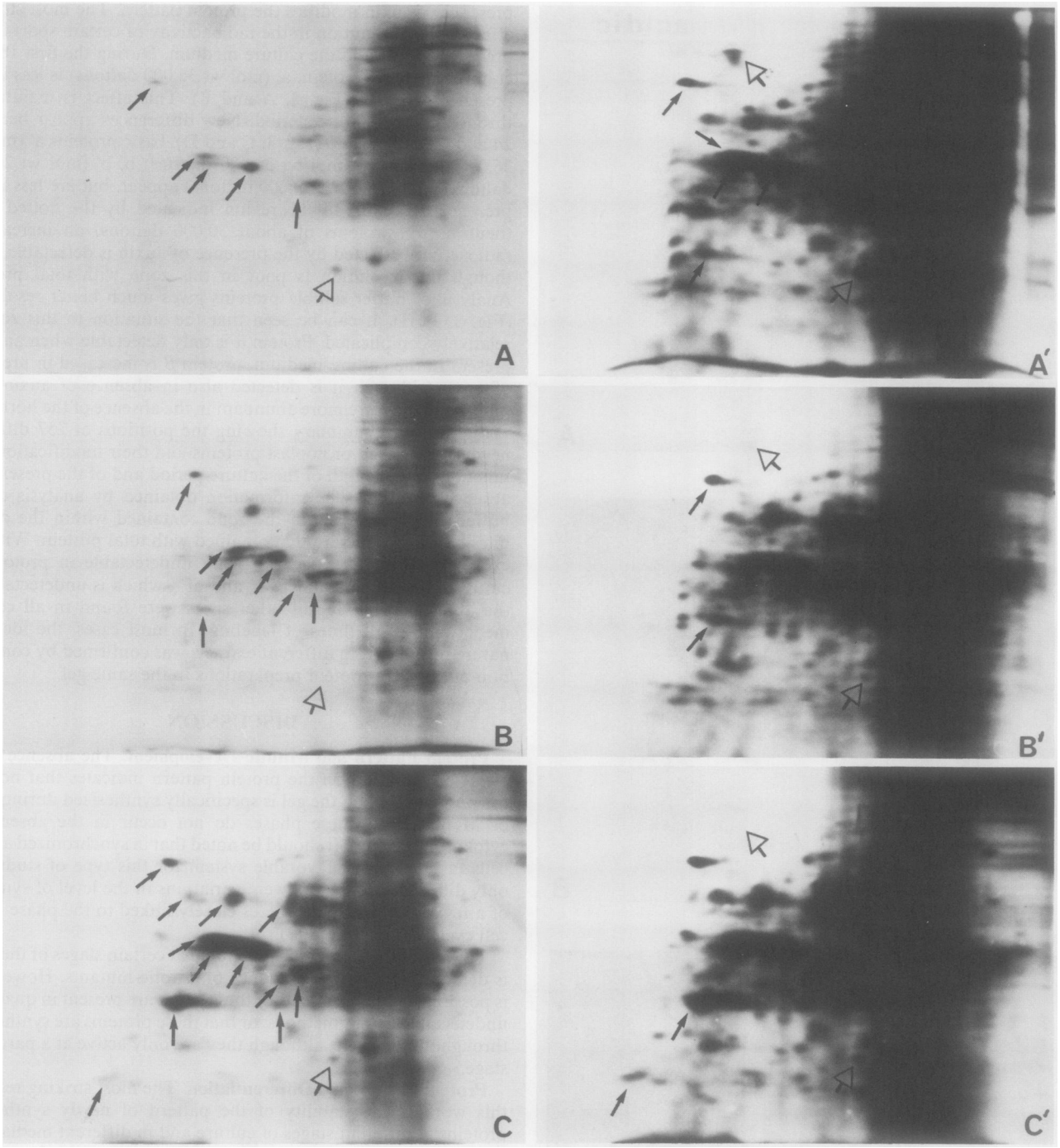


FIG. 2. Development of protein pattern during culture in complete medium. Protoplasts were labeled 0 to 18 hA'), 24 to 42 h (B and B'), and 48 to 66 h (C and C'). Fluorographs were obtained after exposures of 18 h (A, B, C) and 7 days (A', B', C') at −70°C. Closed arrows, spots which increase in intensity or appearing late during culture; open arrows, those which decrease.

culture, etc.). This interpretation is compatible with the observation of Ivarie and O'Farrell (8) who detected variations in the protein pattern of rat hepatoma cells during culture; these variations particularly concerned proteins sensitive to steroids.

Protein Pattern and Hormonal Effects. A consideration of the literature leaves an ambiguous impression. On the one hand, many publications indicate hormonal effects on extremely varied enzyme activities, while on the other hand, the few studies of total protein patterns show either no detectable hormonal effect for auxin (1), or for cytokinin (this work), or an effect limited to a small number of proteins for cytokinin (6, 9), or for auxin and cytokinin (19). In fact, the observed modifications of enzyme activity often occur long after hormone application, frequently in high, nonphysiological concentrations. Further, whereas the hormonal effect on an activity has been published, the absence of an effect on other enzymes has generally not been published. This has led to an overestimate of the impact of hormones on protein synthesis. On the other hand, one can ask whether the resolution of two-dimensional gels allows the detection of enzymes, which are proteins present at low concentrations. A rapid calculation shows that this is possible: most enzymes represent, individually, 10^{-3} to 2×10^{-4} parts of the total protein mass, as their yield in a

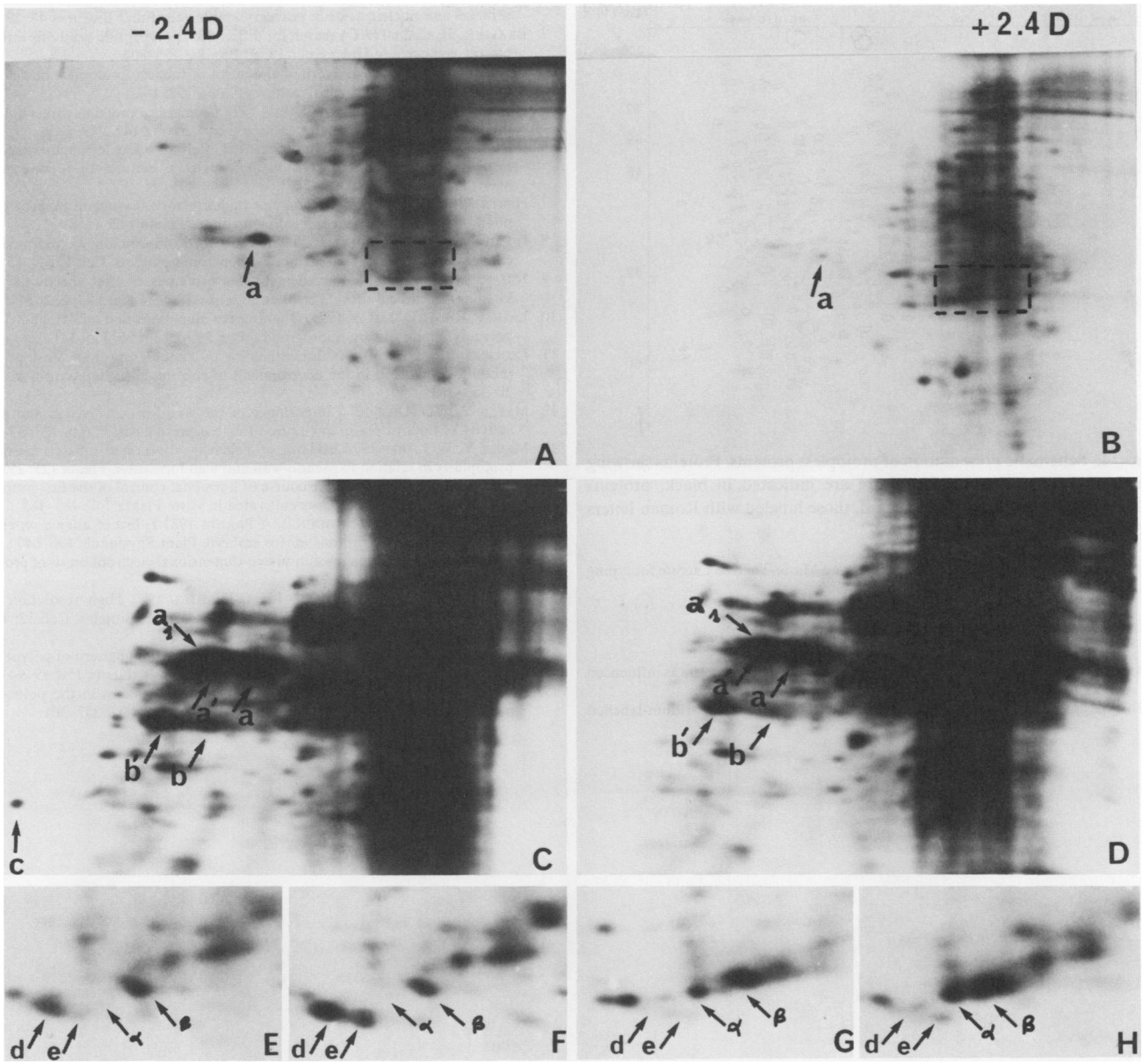


FIG. 3. Hormonal effect on protein pattern. Protoplasts were cultivated in the absence (left side, A, C, E, presence (B, D, G, H) of 2,4-D, with (A, H) or without (B, C, D, E, F, G) N^6-benzyladenine. Radioactive labeling was from 0 to 18 h (A, B, E, G) 24 to 42 h (C, D, F, H); exposure times 18 h (A, B), 2 days (E, F, G, H) and 3 days (C, D). Protein α is only detectable in the presence of the auxin; protein β is increased and proteins a, a', a_1, b, b', c, d, e are decreased by this hormone.

homogeneous state corresponds to purification by factors of 1,000 to 5,000. Analysis of Figure 1A shows that the majority of the radioactivity is present in 25 spots. It follows that a spot containing 4% of the radioactivity is detectable after 2 h exposure. The detection of certain spots requires exposures of 10 days. This corresponds to a 3×10^{-4} part of the total protein mass, which is in the range of most enzymes. It should be emphasized that this precision is only obtained in the least charged regions of the gel where diffusion from heavily labeled spots does not interfere with the detection of minor spots.

Our study of total protein patterns at physiological hormone concentrations and within a short time of hormone application should, therefore, give a truer picture of the hormonal effect on gene expression.

Among the effects of auxin, the induction of spot α is particularly interesting, as it is an all-or-nothing effect and is observed during the first labeling period, *i.e.* relatively soon after hormone application. Future work will determine the minimum period between application of auxin to the protoplasts and the appearance of spot α, as well as establish whether it is a newly synthesized peptide or results from a posttranslational modification of a peptide synthesized in the absence of applied auxin. The presence of α among the buffer-soluble proteins opens the way to the cellular localization of this peptide. This will probably be facilitated by the characteristics of the protoplasts.

The very limited effect of auxin on the protein pattern allows us to resolve the dilemma as to the roles of auxin and isolation in setting in motion the mitotic program of tobacco protoplasts. Although this hormone is necessary from the beginning of the culture, if intervenes only in an early and specific stage, and the isolation of the protoplast from the mesophyll tissue is responsible for the former's meristematic state as characterized by its bidimensional protein pattern.

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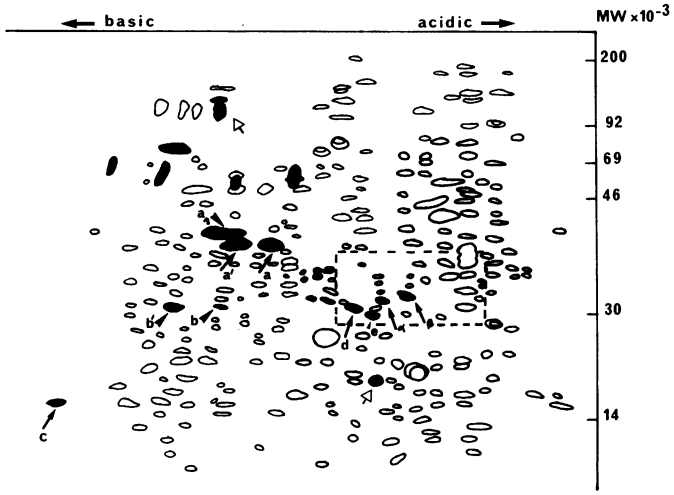

FIG. 4. Schematic presentation of protoplast proteins. Proteins showing a variation in intensity during culture are indicated in black, proteins labeled with Greek letters are increased, those labeled with Roman letters are decreased in presence of 2,4-D.

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