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PROTEIN SYNTHESIS DURING HORMONE STIMULATION IN THE AQUATIC FUNGUS, ACHLYA

C. J. Michalski

Laboratories of Molecular Biology, Faculty of Medicine

Memorial University of Newfoundland

St. John's, Newfoundland, Canada A1B 3V6

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SUMMARY

One hour following administration of physiological concentrations of the steroid hormone antheridiol to a male strain of the water mold, Achlya ambisexualis, the rate of total cellular protein synthesis is increased. Further analysis revealed a sequential increase in the rate of syntheses for three classes of proteins following hormone stimulation. The rate of ribosomal protein synthesis increased as early as 20-30 minutes, followed by ribosomal salt wash proteins (40-60 minutes) and total soluble proteins after 60 minutes. Patterns of total cellular proteins, resolved by two-dimensional gel electrophoresis, during the first four hours after hormone treatment demonstrated the appearance of two newly synthesized peptides beginning at approximately 40 minutes followed by an increased rate of synthesis of three peptides after one hour. The synthesis of two peptides totally decreased after three hours of hormone induction.

INTRODUCTION

Achlya, a genus of the aquatic Phycomycetes, has been found to produce and respond to steroid hormones (1). Antheridiol, a sterol which has been chemically synthesized (2, 3), is secreted by female strains of Achlya and induce the male strain to form its sex organ initials. Once induced the male strain produces and secretes a second hormone, oogoniol, which stimulates the female strain to form its sex organs, the oogonia.

To study the biochemical events associated with hormone induced growth and development, a number of investigators have utilized Achlya as a model system. Recent preliminary evidence has suggested that antheridiol acts via a protein receptor-hormone complex similar to that for mammalian hormones (4). Increased rates of total RNA and total protein synthesis have also been identified as early events following antheridiol stimulation in Achlya (5-8). Whether

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activation of synthesis of particular classes of cellular proteins occurs is not known. Biochemical and cytological data first revealed that the enzyme, cellulase (EC 3.2.1.4), which is involved in cell wall softening at the sites of antheridial branch formation, is activated following hormone treatment (5, 9, 10). It has also been shown that antheridiol stimulated the accumulation of an induced protein of M.W. 69,000 detected by disc gel electrophoresis (11).

This current investigation is designed to (1) determine whether ribosomal proteins and ribosome associated factors are co-ordinately synthesized with respect to total cellular protein and (2) examine the effect on gene expression following antheridiol stimulation in Achlya.

MATERIALS AND METHODS

Chemicals - Achlya ambisexualis strain E87 was supplied by Dr. W. E. Timberlake. Synthetic antheridiol was a generous gift of Dr. T. C. McMorris. Leucine, L-[(4, 5 -^{3}H (N)], 40-60 Ci/mmole, Leucine, L-[^{14}C (U)], 270 Ci/mmole and methionine, L-[^{35}S], 300-400 Ci/mmole were obtained from New England Nuclear. All other chemicals were of reagent grade.

Growth conditions - Achlya ambisexualis J. R. Raper, male strain E87, was used throughout this study. Asexual spore suspensions obtained by the method of Griffin and Breuker (12) were inoculated into liquid PYG medium (13) at a concentration of 2-3x10^{3} viable spores per milliliter of medium and grown for 14 hours at 25C on a gyratory shaker at 200 rpm. A suspension of the above culture was added at a dilution of 1:10 to a culture medium containing L- glutamic acid, monopotassium salt, 2.6mM; D-glucose, 16mM; TRIS buffer, 10 mM; L-methionine, 0.1 mM; KCl, 20 mM; Ca Cl_2, 0.5 mM; KH_2PO_4, 1.5 mM; $FeSO_4$, 4.5 uM; EDTA, 5.0 uM; Mg SO_4, 0.5 mM; micronutrients were according to Griffin et al (14) and the pH adjusted to 6.9 with HCl. The cultures were then grown at 28C for 17 hours on a gyratory shaker at 150 rpm. After this time cultures were treated with synthetic antheridiol, 1.0 ng/ml, dissolved in methanol, or left untreated as controls.

Separation of cellular material - Cultures were harvested on millipore filters, washed with an equal volume of fresh culture medium and mycelia frozen in liquid N_2. Frozen mycelia were ground to a powder with a pestle and transferred to TKM buffer containing 50 mM Tris-HCl, pH 7.2; 10 mM Magnesium acetate; 6mM, 2-mercaptoethanol and 80 mM, KCl. After thawing mycelia were then gently homogenized and centrifuged at 15,000 g for 10 min. The supernatant was retained and served as the source of total soluble protein. Protein determinations of the extracts were performed by the method of Lowry et al(15). Ribosomes were pelleted from the cell-free extracts by centrifugation at 60,000 rpm for two hours. Ribosomal pellets were then resuspended in TKM buffer containing 600 mM KCl, washed for two hours and finally centrifuged for two hours at 60,000 rpm. The above supernatant was retained as the ribosomal salt wash. Ribosomal pellets were then washed once, pelleted and resuspended in TKM buffer.

Two-dimensional gel electrophoresis - Mycelial cultures were induced with antheridiol (1.0 ng/ml) and divided into 20 ml aliquots to which 60 uCi of ^{35}S-methionine was added at 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 min. after hormone administration. An uninduced culture served as a control. Cultures were allowed to take up label for 20 min. after which the mycelia were collected by filtration, frozen in liquid N_2 and ground to a powder. Broken mycelia were then transferred to 300 ul of buffer containing 0.01 M Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 50 ug of pancreatic RNase per ml. The frozen mycelia were thawed in the above buffer and centrifuged for five min. in a microfuge after which 200 ul of the supernatant was treated with 10 ul of stock pancreatic DNase; 1 mg/ml of ultra pure urea was added followed by 225 ul of solution containing 9.5 M urea, 2% (w/v) Nonidet-P40, 2% Ampholines (comprised of 1.6% pH range 5-7 and 0.4% pH range 3-10) and 5% 2-mercapto-ethanol. Two-dimensional gel electrophoresis was according to the method of O'Farrell (16). Approximately 4-5 ug of protein (200-225,000 dpm) was layered on to isoelectrofocusing gels 2.5mm x 13mm. Isoelectrofocusing gels were run in duplicate, one of which was solublized in H_2O_2 and counted in Aquasol (New England Nuclear) to estimate the number of counts entering the gel. The second dimension, 10-16% exponential gradient, SDS slab gels were 10 cm x 14 cm x 1.5 mm thick. Following electrophoresis slab gels were fixed in acetic acid, treated with DMSO (dimethyl sulfoxide) and impregnated with PPO according to the method of Bonner and Laskey (17). Gels were dried, pressed against Royal X-O Mat R, x-ray film and exposed at -80C to the equivalent of 1 x 10^6 cpm for 24 hours.

RESULTS AND DISCUSSION

To determine the rate of total protein synthesis following hormone administration, cultures were incubated with antheridiol (1.0 ng/ml) and 2.0 uCi/ml of [^{3}H] leucine was added at various times following hormone administration. After five minutes the mycelia were filtered, cell extracts obtained and aliquots selected for protein determination and TCA precipitation for liquid scintillation counting. Incorporation of [^{3}H] leucine into protein for non-induced and antheridiol stimulated cultures is illustrated in Fig. 1.

The rate of incorporation for hormone treated cultures remained constant for approximately 40 min. then showed a slight increase before dramatically increasing after 60 min., while control cultures remained constant throughout the labelling period.

The next experiment was designed to determine the influence of antheridiol upon the rates of synthesis of total cellular protein, ribosomal protein, and ribosomal salt wash proteins, which contains ribosome associated factors. Cultures of Achlya were prelabelled with [^{14}C-] leucine (0.1 uCi/ml) and unlabelled leucine (2 ug/ml) and allowed to grow for two hours. Antheridiol was

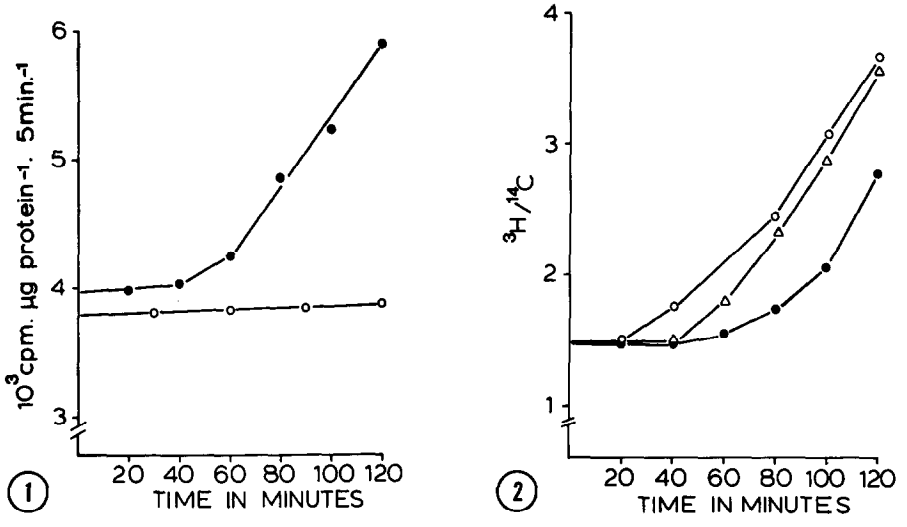

Figure 1. Incorporation of [^{3}H] leucine into cellular protein during hormone induction. Antheridiol was added to cultures at t=0. At times after that cultures were labelled with the addition of 2.0 uCi/ml of [^{3}H] leucine. After five minutes mycelia were collected, cell free extracts secured and aliquots added to hot 10% TCA. Acid precepitable material was collected and millipore filters counted in 1.0 ml Protosol (New England Nuclear) and 10 ml of Omni-fluor - Toluene mixture. Rate of incorporation minus hormone (O—O); plus antheridiol (●—●).

Figure 2. Synthesis of cellular proteins following antheridiol administration. Mycelia were pre-labelled with [^{14}C] leucine for two hours after which 1 ng/ml of antheridiol was added. Cultures were then pulsed with 2.5 uCi/ml of [^{3}H] leucine for five minutes and chased for two hours with 1.5 mg/ml of unlabelled leucine. Extracts were treated as described in Materials and Methods. The $^3H/^{14}C$ ratios of total cell protein (●—●); ribosomal protein (O—O) and ribosomal salt wash proteins (Δ—Δ).

then added and the culture separated into seven flasks containing 120 ml each of the pre-labelled culture. At t=0, 20, 40, 60, 80, 100 and 120 min. the cultures were pulsed for five min. with 2.5 uCi/ml of [^{3}H] leucine then chased with 1.5 mg/ml cold leucine for two hours to allow assembly of labelled ribosomal proteins into mature ribosomes. Cells were then harvested and the ribosomes and ribosomal salt wash obtained as described in the Materials and

TABLE 1

Differential Rates of Synthesis

of Ribosomal Proteins and Ribosomal Salt Wash Proteins

Time after hormone treatment (min.)	$\frac{^3H/^{14}C \text{ Ribosomal Proteins}}{^3H/^{14}C \text{ Total Soluble Protein}}$	$\frac{^3H/^{14}C \text{ Ribosomal Salt Wash}}{^3H/^{14}C \text{ Total Soluble Protein}}$
0	$\frac{1.46}{1.45} = 1.00$	$\frac{1.44}{1.45} = 1.00$
20	$\frac{1.45}{1.44} = 1.00$	$\frac{1.46}{1.44} = 1.01$
40	$\frac{1.74}{1.47} = 1.18$	$\frac{1.49}{1.47} = 1.01$
60	$\frac{1.99}{1.56} = 1.28$	$\frac{1.80}{1.56} = 1.15$
80	$\frac{2.45}{1.75} = 1.40$	$\frac{2.34}{1.75} = 1.34$
100	$\frac{3.08}{2.06} = 1.50$	$\frac{2.89}{2.06} = 1.45$
120	$\frac{3.68}{2.78} = 1.32$	$\frac{3.59}{2.78} = 1.29$

Methods section. $^3H/^{14}C$ ratios were then calculated correcting for spillover, and the rate of synthesis of total soluble protein, ribosomal protein and ribosome associated factors determined as illustrated in Fig. 2. These results indicate that there is an increase in the rate of ribosomal protein synthesis approximately 20 min. after antheridiol stimulation followed by an increase in the rate of ribosome associated factors at 40 min. Synthesis of total cell protein increased after a 60 min. delay. The above three classes of proteins were found to be coordinately synthesized in control experiments without added hormone. Making use of these data, a table is constructed in which the rate of ribosomal protein synthesis and the rate of ribosomal salt wash protein synthesis are expressed as a function of total protein synthesis. Presenting the data in this way eliminates any influence that changes in amino acid pool size might cause. The data, Table 1, indicate that at time 0 following antheridiol

administration, the rates of synthesis of ribosomal proteins and ribosome associated factors expressed as a function of soluble protein formation is equivalent. At times 20 and 40 min. following hormone induction, the relative rates of synthesis of ribosomal and ribosomal salt wash proteins versus protein versus total protein show a variation from 1.

That ribosomal protein synthesis is detected early following hormone stimulation is not surprising since increased ribosomal RNA synthesis has been detected at approximately the same time (7) and immediately precedes the formation of antheridial initials in Achlya. It is interesting to note that ribosome associated factor synthesis shortly follows ribosomal protein synthesis and that both these classes of proteins precede apparent rate increases in total protein synthesis. Similar observations have been made by Sells et al following a nutritional shift-up in bacteria (18) and during liver regeneration (19) suggesting that the above sequence of events may be associated with the increased growth rate phenomenon.

With the dramatic increase in the rate protein synthesis following antheridiol stimulation it is interesting to speculate on the effect that the hormone has on gene expression. Are various genes turned on or off? To what extent is the genome influenced with respect to the number and kinds of proteins affected? Previous attempts to estimate the number of specific proteins induced by antheridiol were limited by the resolving power of single dimension disc gel electrophoresis. Taking advantage of the resolving power of the O'Farrell two-dimensional electrophoresis system and autofluorography, the following experiment was designed to detect alterations in total cell protein patterns following antheridiol treatment. A stock culture of Achlya was divided into 20 ml sub-cultures after the addition of hormone. These cultures were labelled for 20 min. periods with [^{35}S] methionine at t=0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 and 220 min. Mycelia were harvested and cell extracts prepared for two-dimensional gel electrophoresis as described in Materials and Methods. A control culture of non-induced mycelium

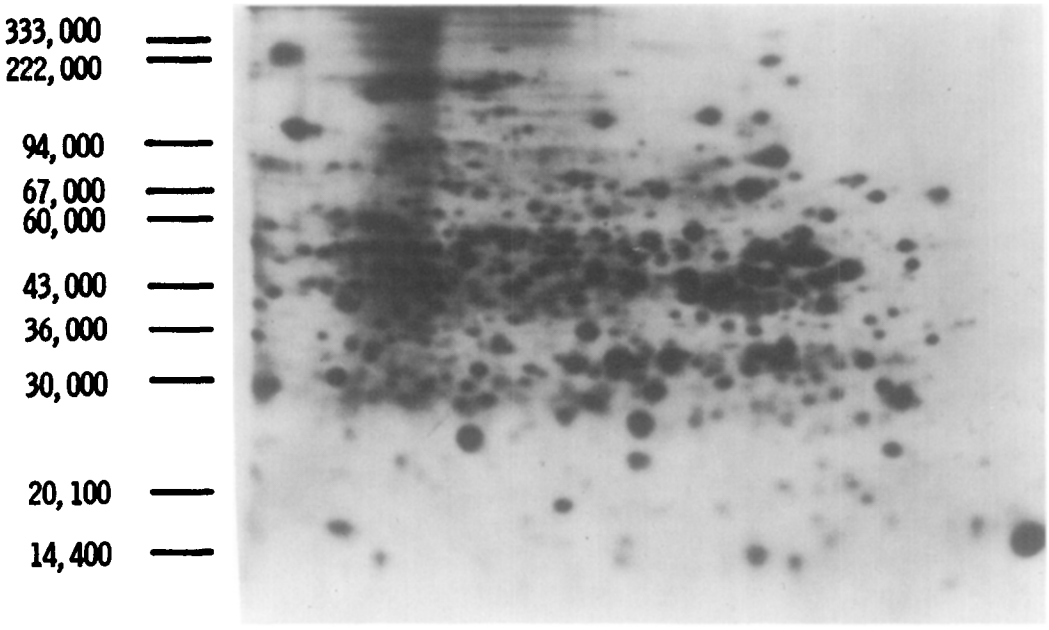

Figure 3. Two-dimensional gel of proteins synthesized by non-induced mycelia. To 20 ml of culture was added 3.0 uCi/ml of [^{35}S] methionine for 20 minutes. Cells were harvested and prepared for electrophoresis as described in Materials and Methods. This gel contained approximately 2 x 10^5 cpm and the fluorograph exposed for five days at -80C. First dimension electrofocusing from pH3 (left) to pH 10 (right); second dimension is electrophoresis on an exponential gradient, 10-16% acrylamide containing 0.10% SDS. Molecular weight markers appear in left margin and are as follows: Thyroglobulin, 330,000; ferritin (half unit) 220,000; phosphorylase b, 94,000; albumin, 67,000; catalase, 60,000; ovalbumin 43,000; lactate dehydrogenase, 36,000; carbonic anhydrase, 30,000; tripsin inhibitor, 20,100; 2-lactalbumin, 14,000.

was also labelled as above and the gel pattern is presented in Fig. 3. Approximately 350 peptides can be resolved in the molecular weight range of 15,000 to 300,000. Following hormone stimulation the earliest detectable change in the cellular protein pattern appears during the 40-60 minute labelling period. At this time the synthesis of two new peptides of molecular weights 85,000 and 25,000 respectively can be detected. After 60 minutes the two peptides are being synthesized at a readily detectable rate (polypeptides

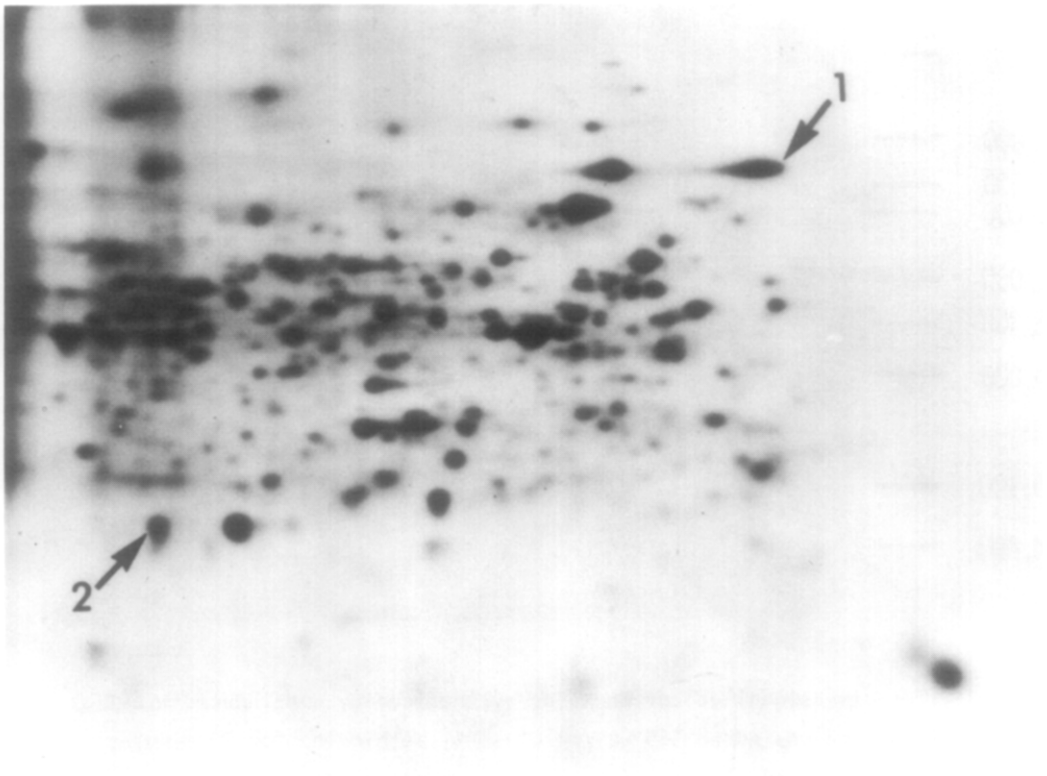

Figure 4. Two-dimensional gel of proteins synthesized 60-80 minutes following antheridiol administration.

1 and 2, Fig. 4). The relative rates of syntheses of three existing peptides also appears to increase at this time (polypeptides 3, 4 and 5, Fig. 5). The spot intensity of these polypeptides of Molecular weights 67,000, 58,000 and 43,000, respectively was readily detected with the naked eye on this underdeveloped gel. The relative increase in the rate of synthesis for polypeptides 3, 4, and 5 was determined to persist up to approximately three hours post hormone induction. After three hours the apparent rates of syntheses for the above proteins leveled off while at the same time the rates of at least two polypeptides, molecular weights 42,000 and 41,000 were dramatically decreased and were no longer detectable (poly-peptides 6 and 7, Fig. 6). Furthermore, the two newly synthesized proteins detected earlier (spots

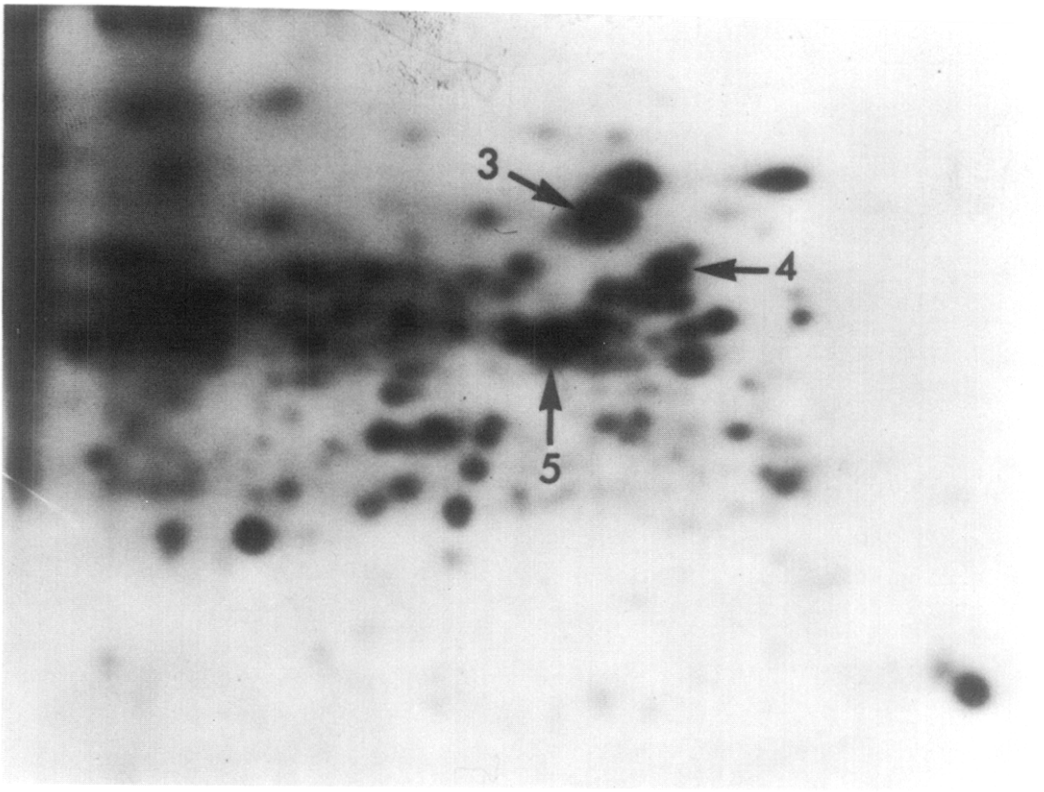

Figure 5. Two-dimensional gel of proteins synthesized 80-100 minutes following antheridiol administration.

1 and 2, Fig. 4) are considerably decreased (spots 1a and 2b, Fig. 6). They were not detectable after 3-1/2 hours post-hormone induction.

A number of limitations are inherent in the above system employed for the analysis of gene products. Of the estimated 2,000 genes expressed in vegetatively growing Achlya cells (18), only 350 polypeptides were detected. This number represents less than 20% of the estimated total and suggests that only those peptides synthesized at a significant rate and those which contained methionine were identified. Furthermore, the changes noted were those consistently visible to the naked eye. Those proteins whose mRNAs exist in very few copies per cell cannot be detected.

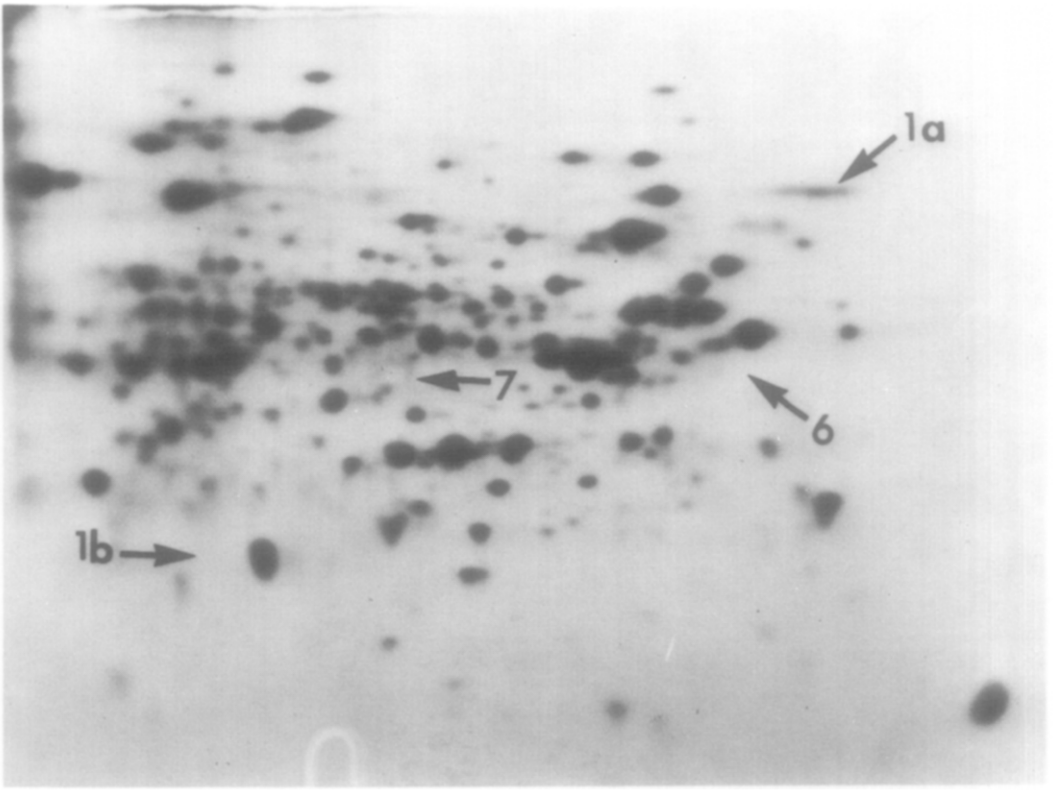

Figure 6. Two-dimensional gel of proteins synthesized 160-180 minutes following antheridiol administration.

It is somewhat surprising that relatively few obvious changes occur with respect to gene expression following hormone stimulation in Achlya. There are potentially 30,000 distinct structural genes in the genome of Achlya, as estimated by Hudspeth, et al (19). Although it has not been determined whether the mechanism of action of antheridiol is similar to that for mammalian hormones, the results presented here suggest that antheridiol induces minor alterations in gene expression. Whether direct binding of antheridiol or an antheridiol-receptor protein complex to the genome affects these changes remains to be determined.

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