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Alterations in soluble protein levels of Neurospora following the addition of galactose were studied by disc electrophoresis on polyacrylamide gels. The isolation of a strain which shows improved growth characteristics on glycerol has allowed the development of a system in which galactose can be used as an inducer under conditions in which glycerol is preferentially utilized as the carbon source. A comparison of lactose and galactose showed lactose to be ineffective as an inducer. Isopropyl-β-D-thiogalactopyranoside did not induce β-galactosidase at the concentrations tested. An attempt to establish gratuitous conditions by isolating a mutant strain which could not metabolize galactose but could be induced to synthesize β-galactosidase was abandoned when the nature of the mutations could not be identified by the techniques available.

Preparations of soluble protein extracted from cultures grown under both induced and noninduced conditions were studied by high resolution electrophoresis. Reproducible differences were observed in induced and noninduced patterns. Beta-galactosidase could not be correlated with a specific protein band due to inactivation during electrophoresis. Enzyme localization studies demonstrated that various enzymes are widely distributed among electrophoretic

protein components and not concentrated in any one band. Observed increases in three protein bands in response to galactose suggest that at least an equal number of proteins are affected. One band has been shown to contain invertase activity. The results are inconsistent with an operon model of regulation in this eucaryotic organism.

ELECTROPHORETIC STUDIES OF INDUCTION IN NEUROSPORA

by

Joyce Lee Barwick

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Approved by

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Date of Examination

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INTRODUCTION

The synthesis of many proteins is known to be influenced by regulatory systems which control the expression of specific structural genes. In many of these systems the presence of a small molecule, known as an inducer, is required for the synthesis of a particular protein. Regulation of protein synthesis in bacteria is relatively clear and has been the subject of many recent review articles (Jacob and Monod, 1961; Maas and McFall, 1964; Epstein and Beckwith, 1968; Cohn, 1957; Halvorson, 1960).

Certain enzymes of microorganisms are formed only when induced by the presence of their specific substrates or closely related structures. The most extensively studied inducible system is the lactose system of the bacterium Escherichia coli. Beta-galactosidase, the enzyme which hydrolyzes lactose and other β-galactosides, requires induction by a galactoside. When E. coli is grown in the absence of a galactoside, very small amounts of the enzyme are produced, but when a galactoside is added to the growth medium, the rate of synthesis may increase 1000-fold with little effect on unrelated enzymes. The inductive response is extremely specific. Only the substrate of the enzyme, or substances closely related in structure to the normal substrate are effective as inducers. Immunological (Cohn, 1957) and

isotopic labeling (Hogness et al., 1955; Rotman and Speigelman, 1954) studies have indicated that addition of inducer brings about the complete de novo synthesis of the enzyme. This induction system and its genetic basis have been thoroughly studied and reviewed by Jacob and Monod (1961).

In bacteria, the activity of genes specifying the structure of enzymes is controlled by the action of specific regulatory genes. The nature of the control systems in higher organisms is not yet understood. The difference between procaryotic and eucaryotic organisms in chromosome structure suggests that the more complex chromosomes of eucaryotic organisms require a more elaborate genetic control mechanism than that found in bacteria.

Bates et al. (1967) have studied the inductive responses of β-galactosidase in Neurospora crassa. Two β-galactosidase enzymes which act on the same substrate have been identified, purified, and characterized. One enzyme shows maximal activity at pH 4.2 while the pH optimum for the other is 7.5. Activities of the two β-galactosidases were found to increase in the presence of lactose or galactose. Induction of Neurospora β-galactosidase was found to differ from bacterial induction in three ways: a longer time was required for response to inducer in the eucaryotic organism; the maximal response was less than that observed in bacteria; the induction of Neurospora β-galactosidase was less sensitive to low concentrations of inducer.

Horowitz et al. (1960) in studying the induction of tyrosinase in Neurospora have found three genes which control the synthesis of this enzyme. One gene determines the structure of the enzyme while the other two genes influence the level of tyrosinase indirectly through their control over the induction mechanism. The appearance and disappearance of tyrosinase during the life cycle is regulated by induction. Aromatic amino acids are effective as inducers. Sulfate acts as a repressor. Tyrosinase is produced constitutively in wild type Neurospora growing on low sulfate medium. The enzyme can be induced when wild type is grown on a medium containing enough sulfate to inhibit constitutive synthesis. Tyrosinaseless mutants ty-1 and ty-2 can be induced to produce the enzyme. In principal the results of Horowitz agree with those of Jacob and Monod in that different genes control the synthesis and regulation of the enzyme. However tyrosinase induction was found to differ from bacterial induction in two ways: (1) The two mutant genes ty-1 and ty-2 are unlike the operator gene of E. coli in that they do not determine whether enzyme synthesis will be constitutive or inductive. (2) The genes are not closely linked, but assort independently.

Eberhart et al. (1964) have studied the β-glucosidase and cellulase activities in Neurospora. There are possibly as many as four enzymes in Neurospora that will attack β-glucosidic linkages. These investigators have isolated a

mutant strain of Neurospora, gluc-1, which is lower in β-glucosidase activity than standard gluc-1^+. The mutation seems to be specifically concerned with aryl-β-glucosidase. Thermolabile β-glucosidase and cellulase activity appear not to be affected by the gluc-1 mutation, while a thermostable glucosidase is greatly reduced in gluc-1 strains. A comparison of gluc-1 and wild type showed no significant differences in physical properties of aryl-β-glucosidase. The gluc-1 gene which apparently has a regulatory function is dominant over gluc-1^+ in heterocaryons containing nuclei of both types. Further studies of these enzymes should give insights into genetic regulatory mechanisms in Neurospora.

Most investigations of regulation of enzyme synthesis have emphasized a single enzyme with little regard for what happens to other proteins. In this study alterations in soluble protein levels following addition of galactose were studied by disc electrophoresis on polyacrylamide gels. The main purpose of this investigation was to determine whether reproducible differences could be observed between the protein patterns obtained from induced and noninduced cultures. Previous electrophoretic studies of Neurospora and related organisms have described certain gross differences not directly related to regulation by induction.

Chang et al. (1962) have used electrophoresis to separate the soluble proteins of Neurospora. Their first separations were obtained on paper. Even with the poor resolution

obtained by this method, the soluble protein could be separated into 6-8 components, and differences could be observed between a wild type strain and a mutant strain. As many as 18 bands could be observed by the starch-gel technique. At least one band was noted as a characteristic difference between the wild and mutant types. The acrylamide gel procedure was found to resolve approximately 25 bands from a soluble protein preparation. The technique was found to be reproducible when applied to the same protein solution and to different protein preparations made from the same genetic strain. They have suggested that with this method for separating and detecting components present in the soluble protein of Neurospora, the synthesis and metabolic significance of these proteins can be investigated as well as changes in the protein complement with genetic constitution.

Steward and Barber (1964) have used acrylamide gel electrophoresis to investigate the genetic basis for differences in the soluble proteins of Neurospora and differences imposed on soluble proteins of the pea plant during growth and morphogenesis.

Gel electrophoresis has yielded distinct and reproducible differences among protein patterns of related species of fungi and of strains within a species (Clare and Zentmyer, 1966; Durbin, 1966; Gottlieb and Hepden, 1966).

Bent (1967) used disc electrophoresis on polyacrylamide gels to separate the soluble proteins extracted from three

Penicillium species. All three could be distinguished by their protein pattern. The pattern and intensity of protein varied greatly with age of culture indicating a need to determine the effects of age and culture conditions before interpreting any protein patterns.

These previous studies suggested the possibility that electrophoresis at high resolution could be combined with specific enzyme assays to provide further information about the process of induction in the eucaryotic organism, Neurospora crassa.

GENERAL PROCEDURES

Strain. The Neurospora strain used in the studies to be described was isolated from reciprocal crosses of 105-L5-A to 74-OR8-1a wild type and selected for ability to grow on glycerol as sole carbon source.

Growth Conditions. Neurospora mycelium was grown in liquid shaking cultures at 30°C. The cultures were grown on 200 ml of Vogel's minimal medium (Vogel, 1956) + appropriate carbon source in 500 ml Erlenmeyer flasks. Vogel's minimal medium and carbon source were autoclaved separately and mixed on cooling. For induced conditions sterile concentrated galactose was added to 34 hour glycerol growing cultures to give a concentration of 0.05 M galactose.

Neurospora stocks were maintained at 5°C on Vogel's minimal medium containing 1.5% sucrose and 1.5% agar.

The conidial inoculum was grown in 250 ml Erlenmeyer flasks on Vogel's minimal medium containing 1.5% sucrose and 1.5% agar. The flasks were incubated at 30°C for 4 days and then placed at room temperature for the remainder of the time prior to inoculation. Conidia used to inoculate the liquid medium were approximately 10 days old. The conidia were harvested with 30 ml of sterile water and filtered through four layers of sterile gauze to remove mycelial fragments. They were counted on a hemacytometer to determine

the volume of conidial suspension necessary to give approximately 2 x 10^8 viable conidia for 200 ml of liquid medium. Cultures were grown for 48 hours. To establish growth curves, flasks were harvested at various intervals. The cultures were harvested on a Buchner funnel, washed with cold deionized water, and blotted dry. The mycelial pads were weighed and then frozen.

Protein Extraction. Soluble protein was extracted with an Omni mixer by homogenizing 0.2 grams of frozen mycelium in 3.0 ml of 0.01 M sodium phosphate buffer, pH 7.5, with 1.0 gram of acid washed glass beads at a speed of 12,570 rpm. The extracted samples were shaken gently in ice for 1 hour on a reciprocal shaker and then centrifuged at 15,000 rpm at 2°C for 20 minutes. The supernate was withdrawn and used for protein and enzyme assays.

This same procedure was used to prepare concentrated crude extracts by extracting 1.2 grams of frozen mycelium in 3 ml of buffer. These extracts were used for electrophoresis. To remove structural protein which interfered with electrophoretic separations, heat supernates were prepared from the concentrated crude extracts by heating the extracts for 15 minutes at 54°C in a water bath shaker, shaking in ice on a reciprocal shaker for 1 hour, and centrifuging for 20 minutes at 15,000 rpm at 2°C. The supernate was withdrawn, assayed for protein content, and used for

electrophoretic separations.

Assays. Total protein content was determined by the biuret method of Gornall et al. (1949). Bovine serum albumin was used as standard.

The β-galactosidase enzymes were assayed by their ability to release o-nitrophenol from the substrate o-nitrophenyl-β-D-galactopyranoside. The pH 4.2 enzyme was assayed by incubating 0.5 ml of crude extract with 2.5 ml of 0.075 M sodium acetate buffer, pH 4.2, and 0.5 ml of 0.01 M ONPG for 10 minutes. The reaction was initiated by the addition of crude extract and terminated by the addition of 1 ml of 1 M sodium carbonate. The tubes were chilled in ice and absorbance was measured by reading at 420mμ on a double-beam spectrophotometer. Corrections were made for substrate and sample blanks.

The pH 7.5 enzyme was assayed by incubating 0.3 ml of crude extract with 1.5 ml of 0.01 M sodium phosphate buffer, pH 7.5 for 10 minutes at 30°C. The reaction was initiated by the addition of crude extract and after 10 minutes of incubation, the reaction was terminated by placing the tubes in ice and adding 1 ml of 1 M sodium carbonate. Absorbance was measured at 420 mμ on a double-beam spectrophotometer. Corrections were made for substrate and sample blanks.

Galactokinase was assayed by the method of Leloir and Trucco (1955). The samples were incubated with ATP and

galactose. At the termination of the reaction, galactose phosphate was precipitated with zinc sulfate and barium hydroxide. The supernate was then assayed by the Nelson procedure (1944) to estimate the amount of reducing sugar present. For controls the same mixture as the experimental samples was incubated without galactose. Galactose was added after addition of zinc sulfate. The difference in reducing sugar between experimental and control samples was taken as the amount of galactose phosphorylated.

Electrophoresis. Electrophoresis was performed by polyacrylamide gel disc electrophoresis as described by Davis (1964). A standard 7% gel was routinely used. These gels were polymerized in two layers in glass tubes of uniform size. The optimal protein concentration of Neurospora extracts for obtaining reproducible densitometric tracings was found to be 0.33 mg. Crude extract samples were diluted with 40% sucrose so that 0.33 mg of protein was contained in a volume of 0.2 ml which was the volume applied to each gel. The tubes were mounted vertically in the electrophoretic apparatus with each end immersed in tris-glycine buffer, pH 8.3-8.4. Both the upper and lower buffer reservoirs were cooled with frozen buffer cubes. Brom phenol blue was added to the buffer in the upper reservoir. Electrophoresis was performed using a voltage regulated power supply with a current of 3 2/3 ma/tube until the brom phenol blue dye had migrated a distance of 42 mm into the lower gel. It

was important that this distance be exact so that protein positions would correspond. For these conditions electrophoresis required approximately 50 minutes. At the termination of electrophoresis, the gels were removed from their tubes by rimming them at each end with cold buffer from a hypodermic needle and syringe. Care was taken to remove all the gels intact and without scarring. After removal from the tubes the gels were immediately stained with 1% amido black in 7% acetic acid or stained for specific enzyme activity. After staining for 1 hour in amido black the gels were destained electrically or by leaching. Destaining was complete when the region of the gel which contained no protein was free of stain. The gels were quantitatively analyzed with a recording densitometer.

Detection of Enzymes in Gels. Dehydrogenase enzymes were detected by the method of Boutwell and Chapman (1966). The gels were incubated in the following solution: 100 ml of 0.3 M tris-HCl buffer, pH 7.4, 100 mg diphosphopyridine nucleotide, 40 mg nitro blue tetrazolium, 2.5 mg phenazine methosulfate, and 50 ml of appropriate substrate (0.4 M sodium lactate, 0.4 M malic acid, 0.4 M glutamic acid, or 0.4 M succinic acid). Incubation for approximately 1 hour at 37°C in the dark was required for the bands to develop clearly. The gels were washed in 7% acetic acid. Migration distance of the bands was measured and the gels were scanned on a recording densitometer.

Alkaline phosphatase was detected by incubating the gel in a mixture of p-nitrophenyl phosphate disodium tetrahydrate and 0.1 M tris-HCl buffer, pH 8.6, in a ratio of 0.5 ml PNP per ml of buffer, at 30°C. The location of the enzyme was revealed within 5 minutes by the appearance of a yellow band which rapidly diffused. Migration distance was measured. Because of the rapid diffusion the gels could not be scanned.

Invertase activity was detected by slicing the gel into 1 mm slices and extracting the protein from the gel slices. Each slice was placed in a small test tube containing 0.3 ml of 1% sodium chloride. The tubes were shaken for 2 hours on a reciprocal shaker. To assay for invertase, 0.2 ml of protein extract in sodium chloride was incubated with 0.2 ml of 0.05 M sodium acetate buffer, pH 5, and 0.1 ml of sucrose (85.5 mg/ml) for 15 minutes at 37°C. To terminate the reaction, the extract was inactivated by heating 1 minute in boiling water. Glucose was estimated by the Nelson procedure (Nelson, 1944).

The pH 7.5 β-galactosidase enzyme was assayed by incubating the gel in a mixture of o-nitrophenyl-β-D-galactopyranoside and 0.01 M sodium phosphate buffer, pH 7.5, in a ratio of 0.3 ml of ONPG to 1.5 ml of buffer at 30°C. The same procedure was used to assay for the pH 4.2 enzyme by substituting 0.075 M sodium acetate buffer, pH 4.2, for the buffer used above.

EXPERIMENTAL

In previous studies suitable growth conditions were established for studying the induction of two β-galactosidase enzymes in Neurospora. Because wild types of Neurospora do not grow well on glycerol, a strain was isolated and selected for ability to grow on glycerol as sole carbon source. This strain, designated 411-L5-A, was used in all the studies to be described.

Concentrations of glycerol varying from 0.10 M to 0.25 M were tested to determine the optimal for growth. Because no significant increase was obtained with an increase in glycerol concentration, a concentration of 0.1 M was selected and used in the early studies. This concentration was increased to 0.18 M in later studies to provide an energy source over a longer period of time.

Galactose, lactose, and isopropyl-β-D-thiogalacto-pyranoside (IPTG) were tested in combination with 0.18 M glycerol for effectiveness as inducers of the two enzymes. IPTG, the most effective inducer of β-galactosidase in Escherichia coli, produced no detectable induction of either enzyme in Neurospora at concentrations as high as 0.05 M. A wide range of galactose concentrations varying from 0.03 M to 0.10 M were found to induce both enzymes. Galactose induces maximal levels of both enzymes at a concentration

of approximately 0.05 M. This concentration was selected to use routinely. Figure 1 shows a comparison of growth curves obtained for 411-L5-A grown on 0.18 M glycerol alone and on 0.18 M glycerol with 0.05 M galactose and with 0.05 M lactose added after 34 hours growth. Cultures were harvested at various intervals after inoculation. The curves represent total growth expressed in grams (wet weight).

When Neurospora is grown on glycerol alone, low levels of both enzymes are produced. When galactose is added at 34 hours, induction reaches a maximum about 12 hours later. When lactose was substituted for galactose under the same conditions, no inductive response of either enzyme was detected. Figure 2 shows pH 7.5 β-galactosidase activity for cultures grown on glycerol, glycerol + galactose, and glycerol + lactose.

In this system galactose can be used under conditions in which glycerol is preferentially utilized as the carbon source. Complications arise when enzyme synthesis is studied under conditions in which the inducer can be metabolized. To overcome any nonspecific secondary effects of the inducer on cell metabolism, Monod and Cohn (1952) have emphasized the use of gratuitous conditions. Gratuitous conditions could not be established in Neurospora by inducing the enzymes with a non-metabolizable galactoside, IPTG. An attempt was made to isolate a mutant strain of Neurospora which could not metabolize galactose yet could be induced to synthesize the

Figure 1. Growth Curves Obtained for Neurospora under Induced and Noninduced Conditions

+ Glycerol

• Glycerol + Galactose

∘ Glycerol + Lactose

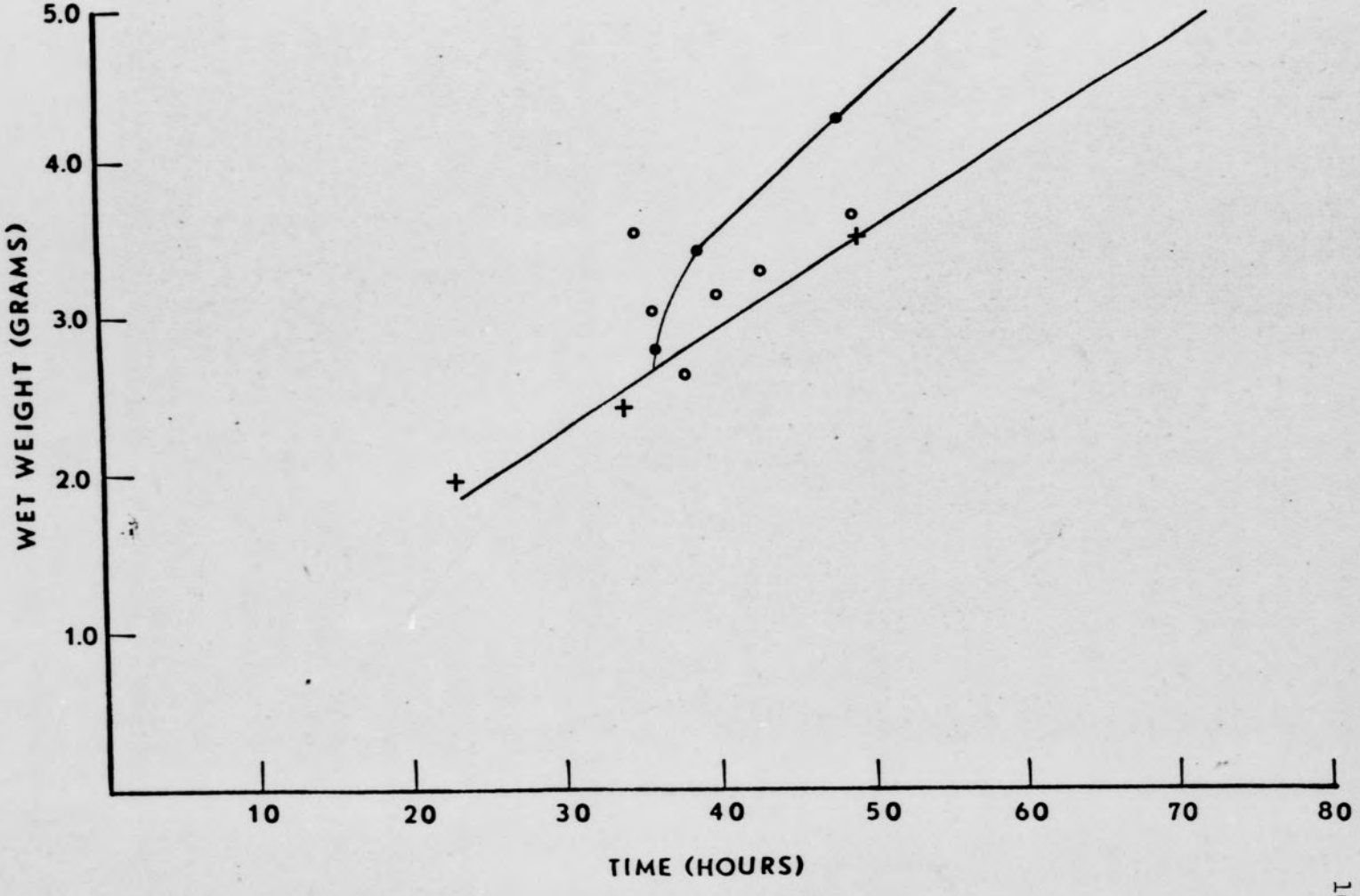
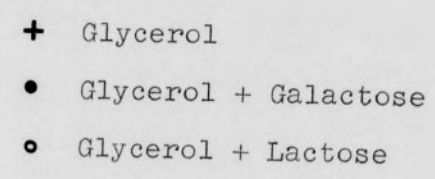
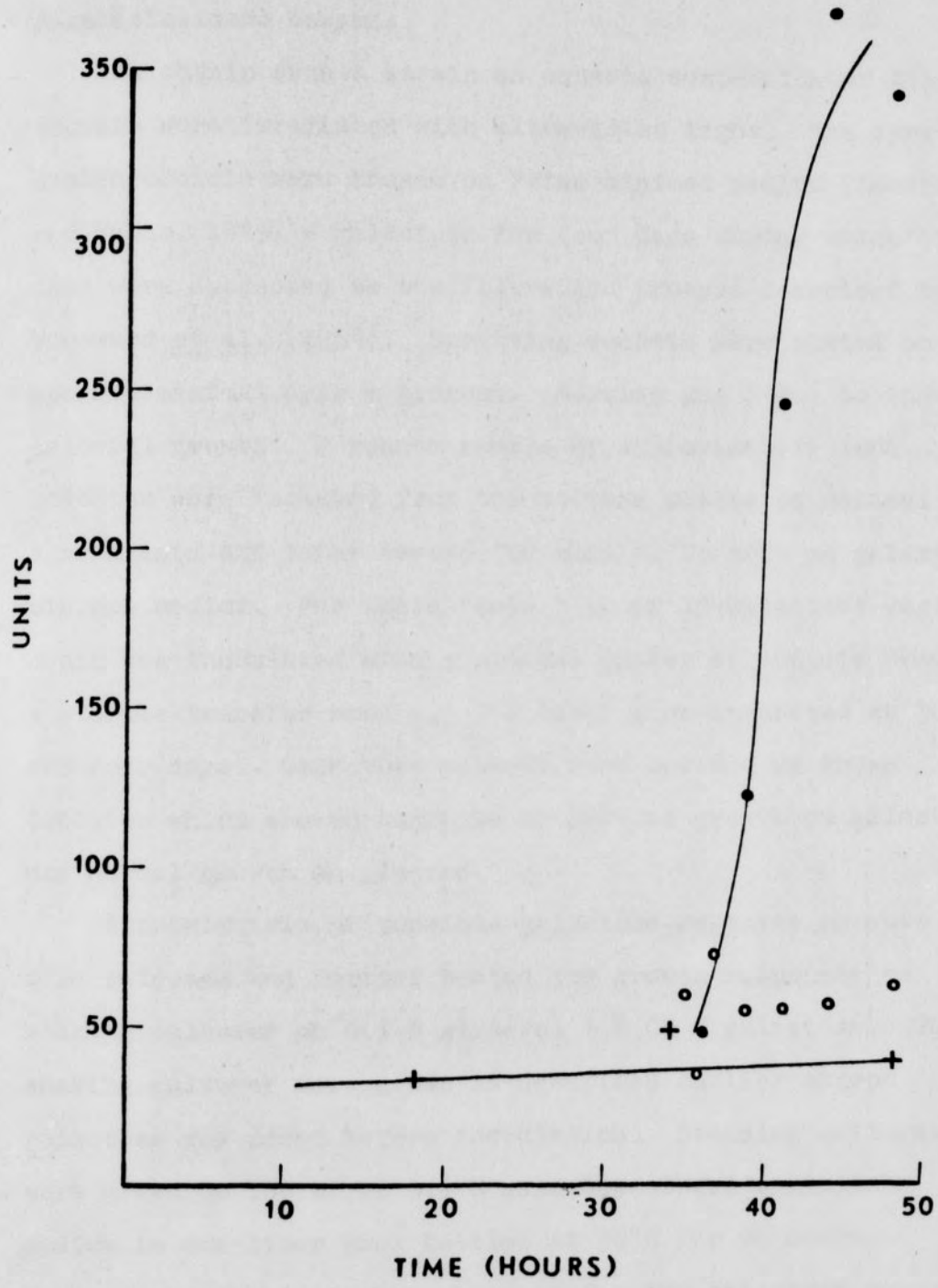

5.0
4.0
3.0
2.0
1.0
WET WEIGHT (GRAMS)
10
20
30
40
50
60
70
80
TIME (HOURS)

Figure 2. Activity of Neurospora pH 7.5 β-galactosidase as a Function of Time



One Unit of Activity = 1000 x Absorbance
(from pH 7.5 enzyme assay)


350
300
250
200
150
100
50
UNITS
10
20
30
40
50
TIME (HOURS)

β-galactosidase enzymes.

To obtain such a strain an aqueous suspension of 411-L5-A conidia were irradiated with ultraviolet light. The irradiated conidia were shaken on Fries minimal medium (Beadle and Tatum, 1945) + galactose for four days during which time they were subjected to the filtration process described by Woodward et al. (1954). Surviving conidia were plated on sorbose minimal agar + glucose. Sorbose was added to induce colonial growth. A random sample of approximately 1000 colonies were isolated from the sorbose plates to minimal agar slants and later tested for ability to grow on galactose minimal medium. For these tests 1 ml of 1% galactose-Vogel's media was inoculated with a minimal number of conidia from a sterile transfer needle. The tubes were incubated at 30°C for four days. Galactose mutants were defined as those isolates which showed negative or reduced growth on galactose and normal growth on glucose.

Approximately 50 possible galactose-negative mutants were selected and further tested for growth responses as shaking cultures on 0.1 M glycerol + 0.05 M galactose. The shaking cultures were grown as described earlier except galactose was added before inoculation. Standing cultures were grown on 100 ml of 0.1 M galactose-Vogel's minimal medium in one liter Roux bottles at 30°C for 96 hours. Table 1 shows the results obtained for the galactose-negative mutants selected for further studies and 411-L5-A for

Table 1

Growth Responses of Galactose-Negative Mutants

Isolate Number	Total Growth (Grams) Glycerol + Galactose	Total Growth (Grams) Galactose
411-L5-A	4.91	1.00
G-I-1	3.87	0.45
G-I-2	5.25	0.02
G-I-4	3.15	0.10
G-I-8	0.60	0.05
G-IV-3	3.15	0.09
G-V-11	3.10	0.10
G-V-13	4.58	0.45
G-VI-1	5.50	0.35
G-VI-6	4.90	0.35
G-VIII-6	5.60	1.15
G-VIII-7	3.40	0.36

comparison. A sample of the media before inoculation and of the filtrate after harvesting was withdrawn and later assayed by the Nelson procedure (Nelson, 1944) for galactose concentration to estimate the extent of galactose metabolism. No correlation could be made between strains unable to grow on galactose and removal of galactose from the medium.

The next experiments were designed to determine why the isolates selected could not metabolize galactose. The first step in the metabolism of galactose is phosphorylation by galactokinase to form galactose-1-phosphate. Extracts of 411-L5-A grown on 2% sucrose and on 0.01 M glycerol + 0.05 M galactose were assayed by the method of Leloir and Trucco (1955) for galactokinase activity. The same procedure was used to assay for hexokinase by substituting glucose for galactose as substrate. By this procedure no significant differences were observed between the experimental and control samples. A further attempt to detect galactokinase activity in 411-L5-A was made by applying the spectrophotometric procedure of Heinrich and Howard (1966). The rate of ADP formation by galactokinase was measured in the presence of pyruvate kinase and lactic dehydrogenase by following the oxidation of DPNH at 340 mμ. When extracts of 411-L5-A grown on sucrose and glycerol + galactose were assayed for both galactokinase and hexokinase activity by this technique, neither enzyme activity could be detected. No further studies of galactokinase were undertaken since the most

sensitive techniques involved the uses of radioisotopes and the necessary equipment was not available at this time.

Crude extracts made from mycelium grown under both induced and noninduced conditions were studied by high resolution electrophoresis to determine the effects of addition of galactose on Neurospora proteins. Extracts of the soluble proteins were subjected to disc electrophoresis on polyacrylamide gels and the stained gels were quantitatively characterized with a recording densitometer.

A large amount of protein was present in crude extracts which apparently aggregated during electrophoresis and interfered with the detection of other proteins. This material was removed by heat precipitation and the supernate was used for electrophoresis. Comparative studies indicated that all the information available from total extracts was also available from heat supernates. The heat supernates allowed better resolution in the quantitative analysis of the electrophoretic separations. Figure 3 shows a comparison of a crude extract and a heat supernate.

Early studies indicated that variations in the electrophoretic patterns which might be attributed to induction by galactose were actually due to changes that occurred with aging of the culture. Figures 4 and 5 show variations observed in extracts made from cultures of two different ages. Figure 4 represents a densitometric tracing obtained from a heat supernate of a 32 hour glycerol + galactose culture.

Figure 5 represents a tracing of a 48 hour glycerol + galactose culture. Galactose was added before inoculation. These variations indicate the necessity of following standardized conditions for growth and extraction of the cultures in order to obtain reproducible patterns for comparison.

To determine the effect of addition of galactose on Neurospora proteins, cultures were harvested at the time of addition and at various intervals thereafter. Figure 6 represents a densitometric tracing of a heat supernate from a 34 hour glycerol culture. Figures 7, 8, and 9 represent tracings of samples taken at 36, 42, and 49 hours after the addition of galactose. Figure 10 represents a 49 hour glycerol sample for comparison.

A comparison of the 49 hour glycerol tracing (Figure 10) and the 49 hour glycerol + galactose tracing (Figure 9) shows the differences in the occurrence of proteins under induced and noninduced conditions. In comparing the two conditions, the induced preparations show increases in at least three peaks after the addition of galactose as well as other minor differences.

The next experiments were designed to determine the location of specific enzymes in relation to the protein bands produced under induced conditions. The enzyme of primary interest, β-galactosidase, is inactivated during electrophoresis and could not be identified in the gels.

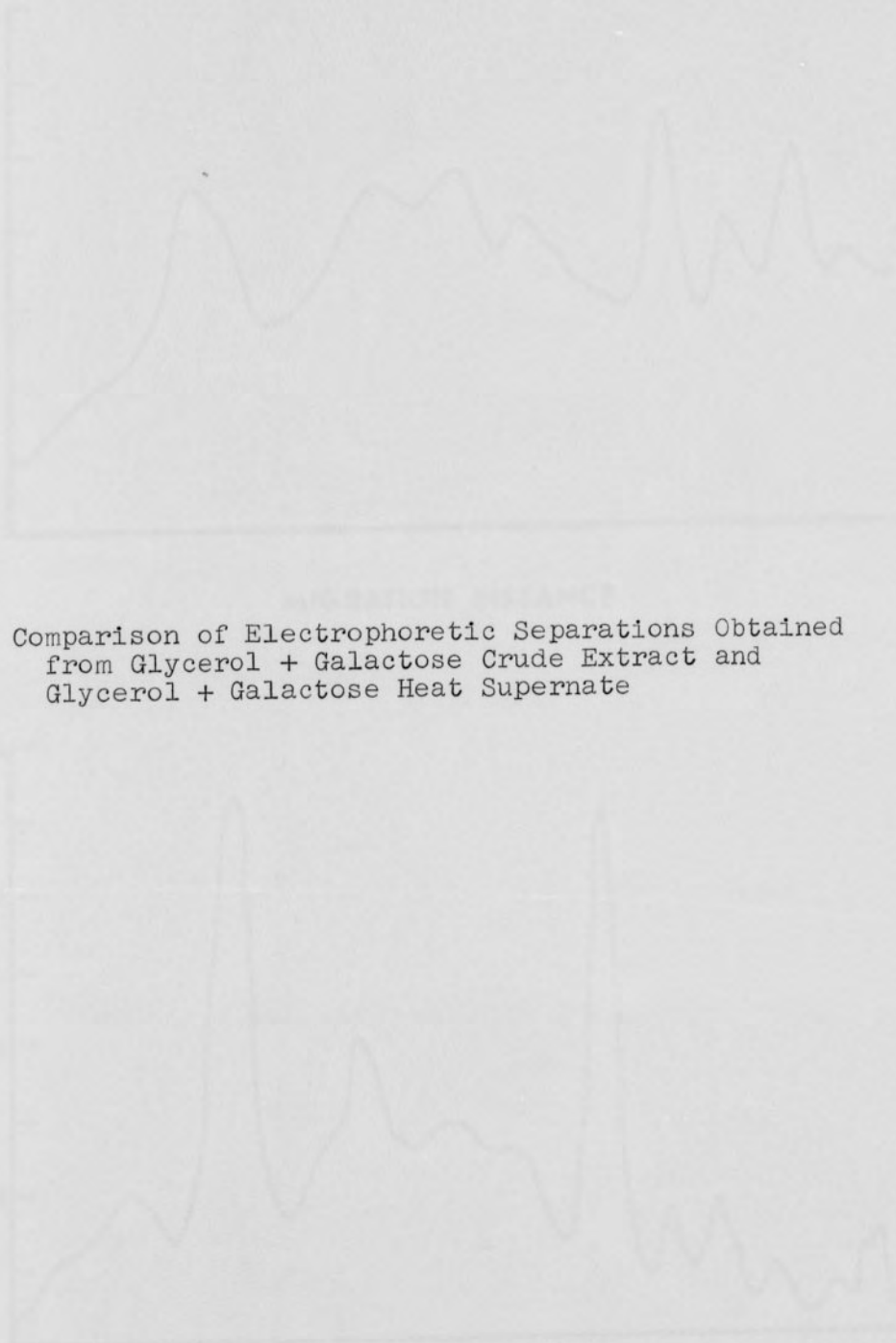
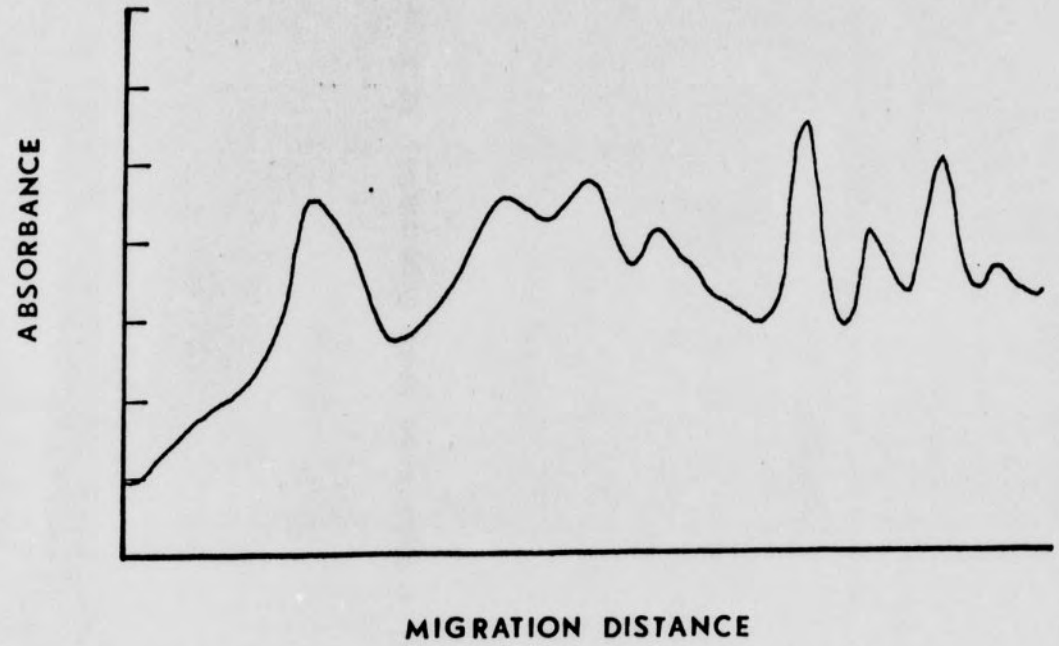
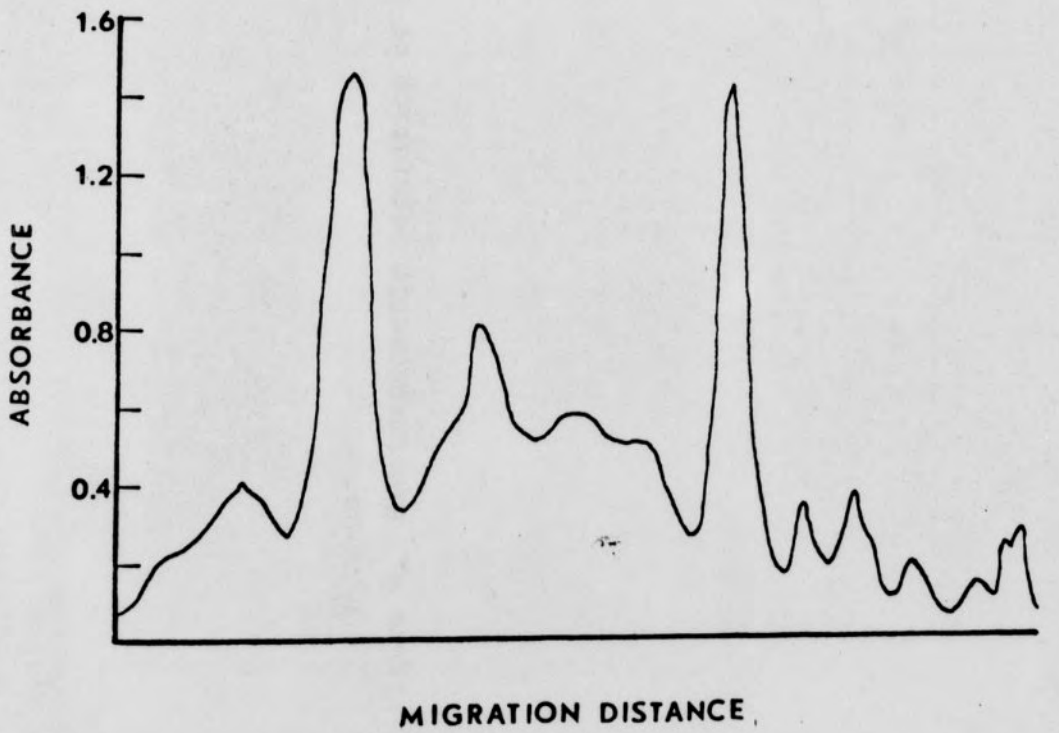


Figure 3. Comparison of Electrophoretic Separations Obtained from Glycerol + Galactose Crude Extract and Glycerol + Galactose Heat Supernate


ABSORBANCE
MIGRATION DISTANCE


1.6
1.2
0.8
0.4
ABSORBANCE
MIGRATION DISTANCE

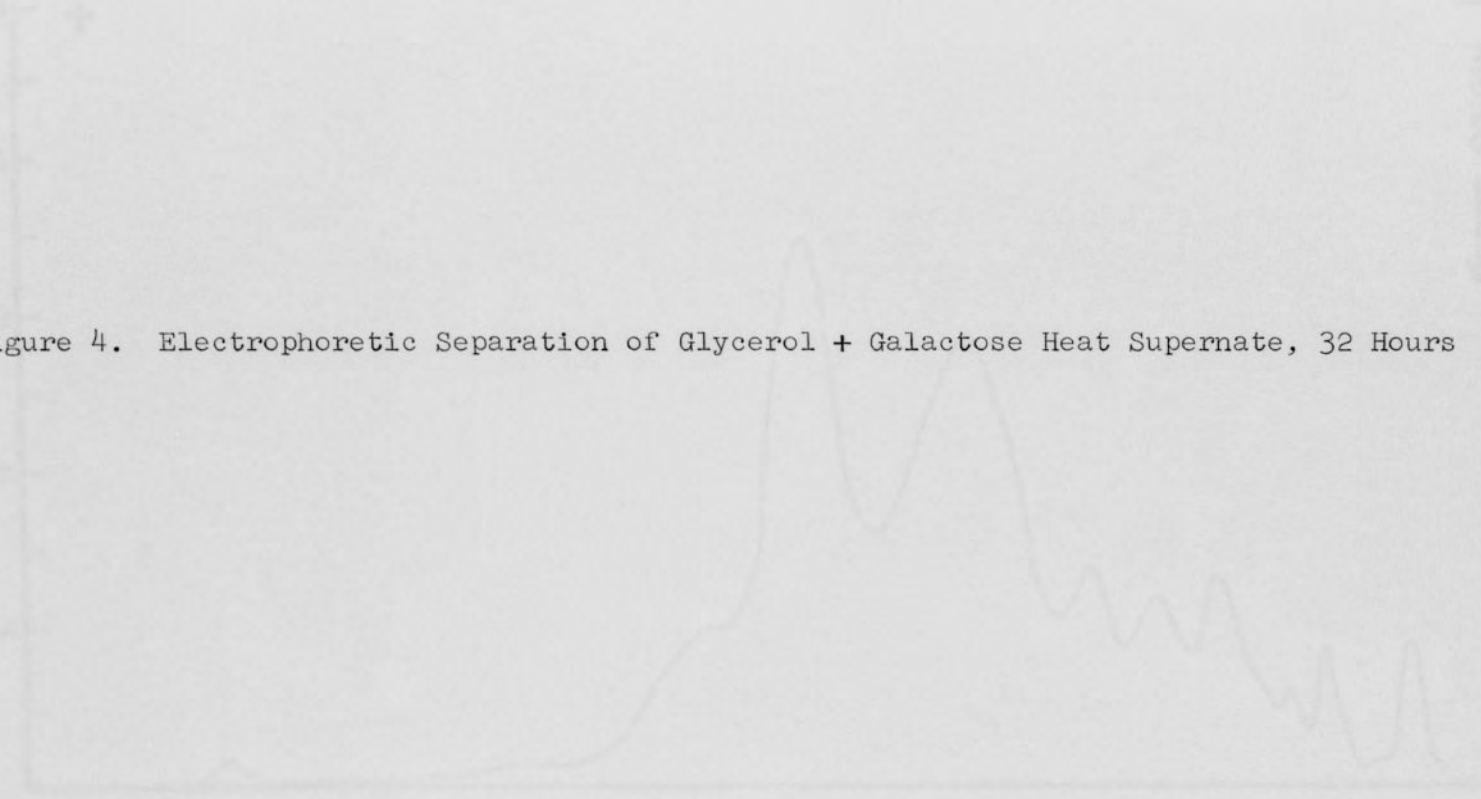


Figure 4. Electrophoretic Separation of Glycerol + Galactose Heat Supernate, 32 Hours

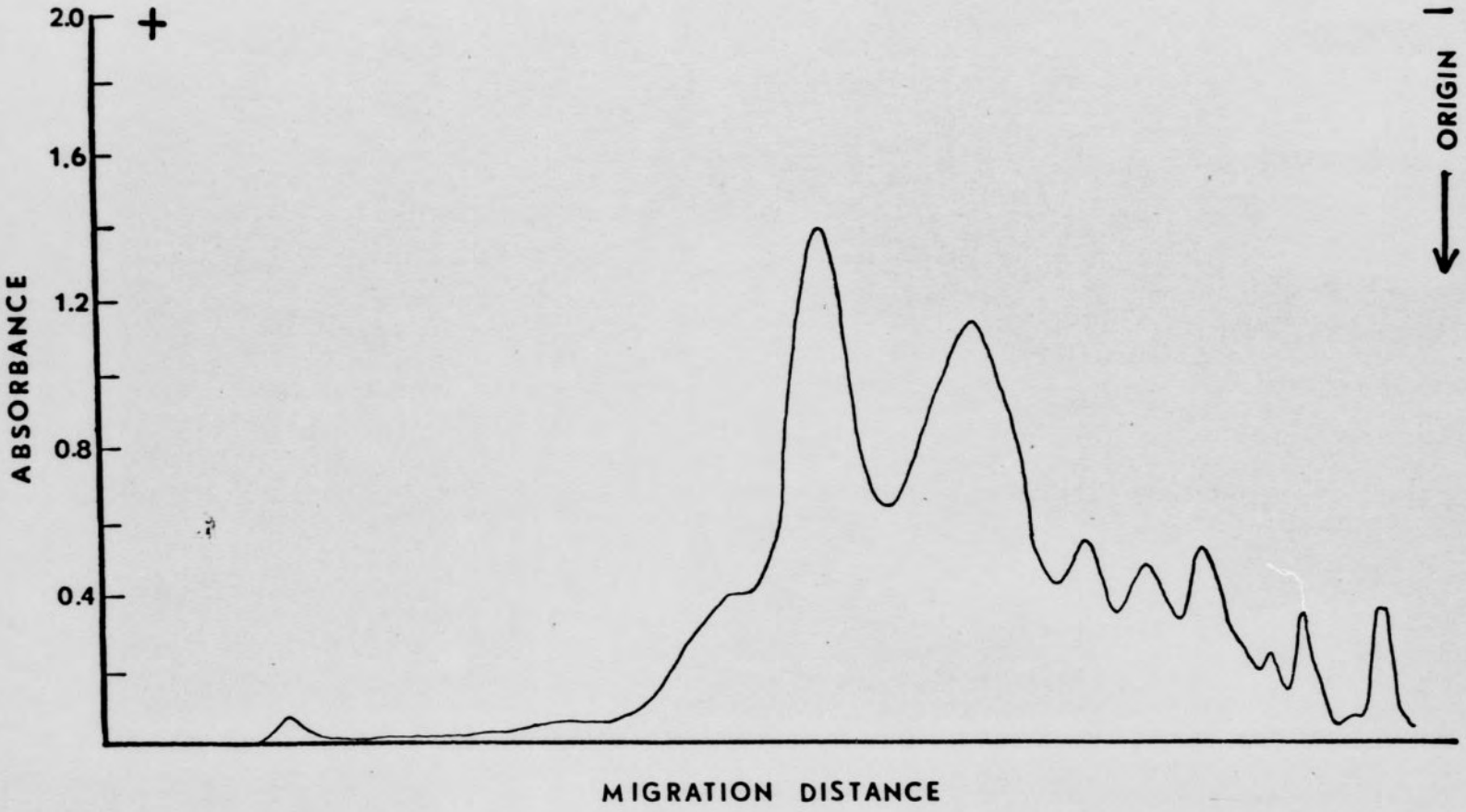

2.0
1.6
1.2
0.8
0.4
+
ABSORBANCE
ORIGIN
MIGRATION DISTANCE

Figure 5. Electrophoretic Separation of Glycerol + Galactose Heat Supernate, 48 Hours

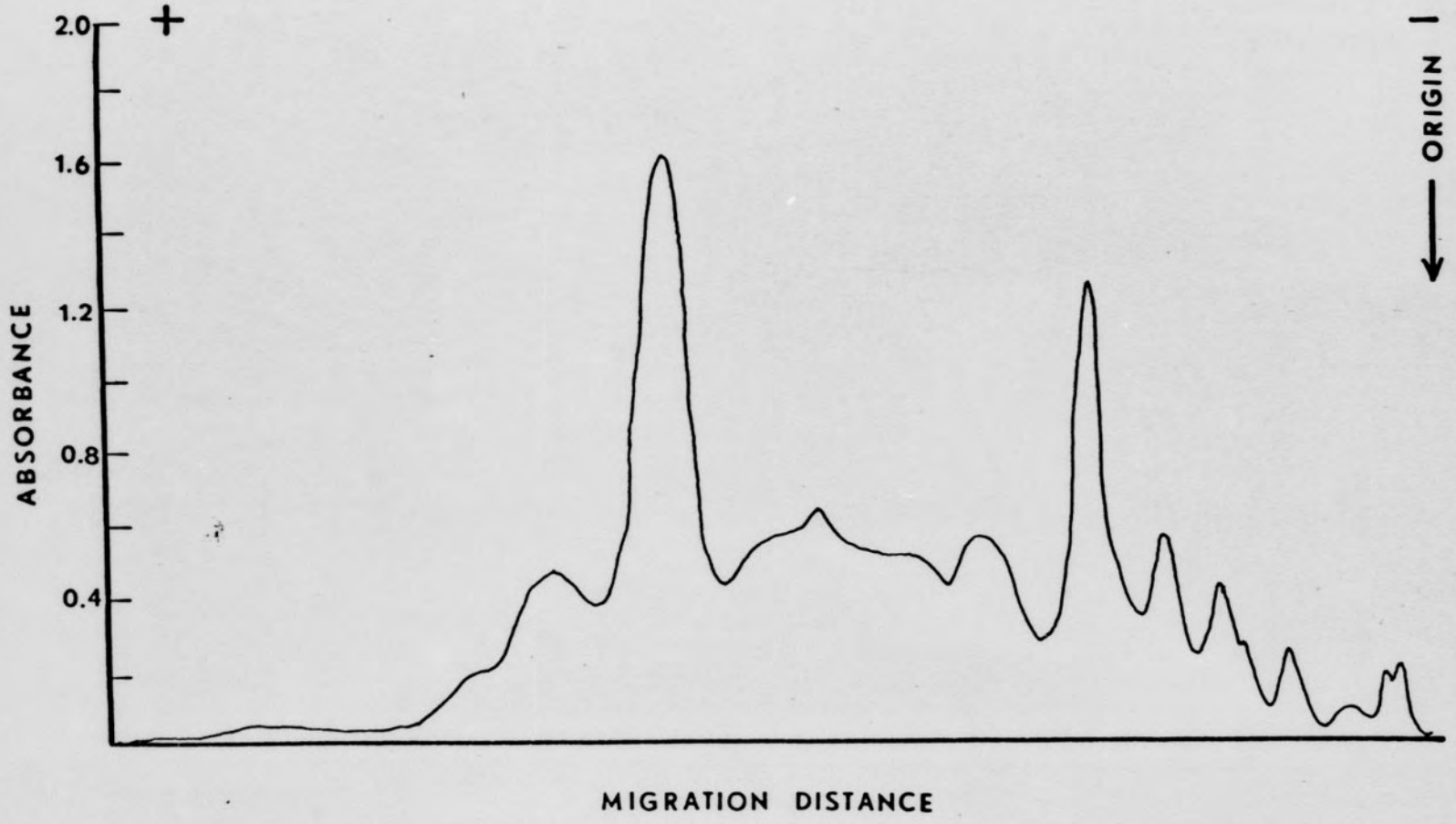

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Figure 6. Electrophoretic Separation of Glycerol + Galactose Heat Supernate, 34 Hours

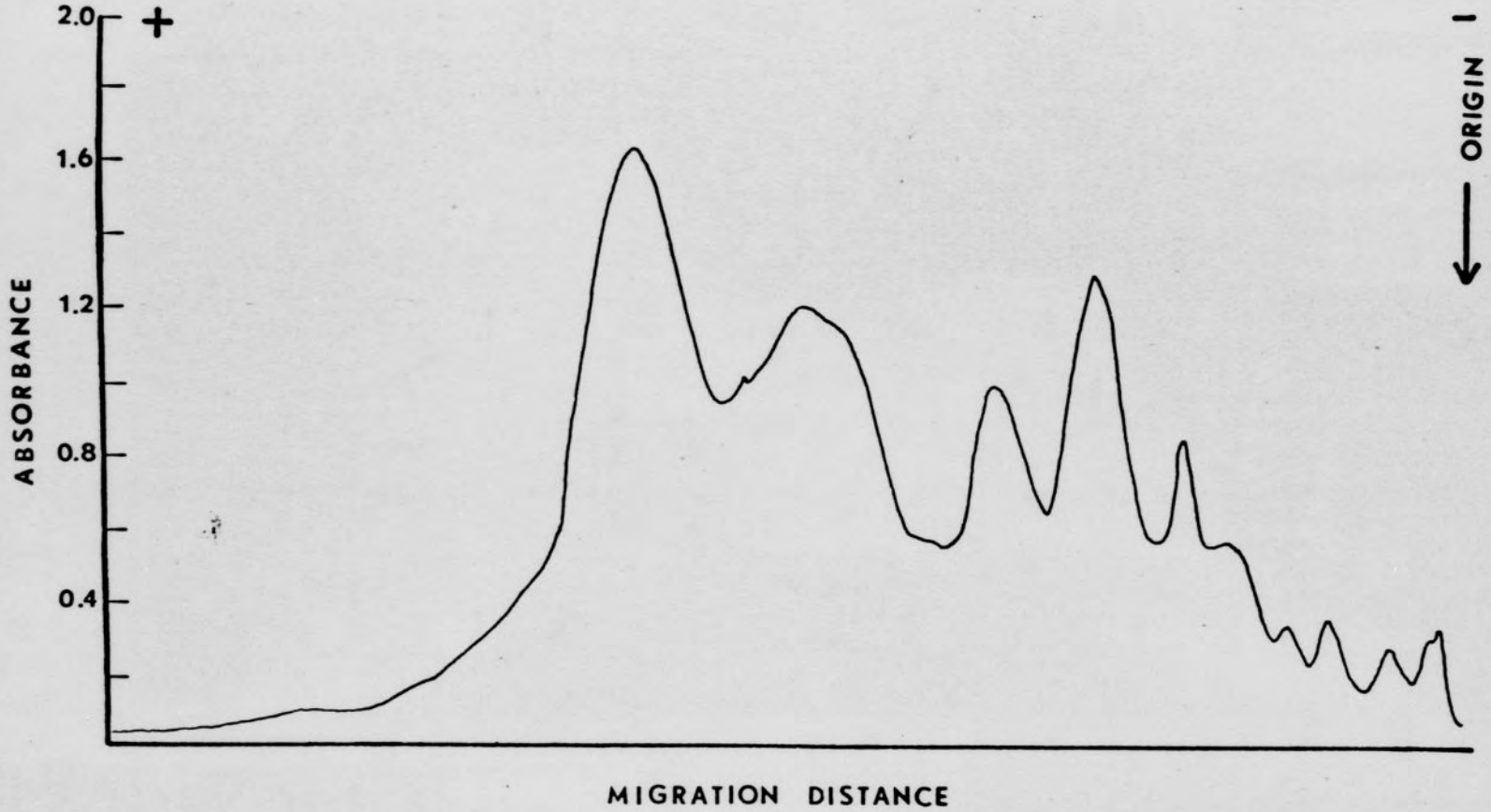
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Figure 7. Electrophoretic Separation of Glycerol + Galactose Heat Supernate, 36 Hours

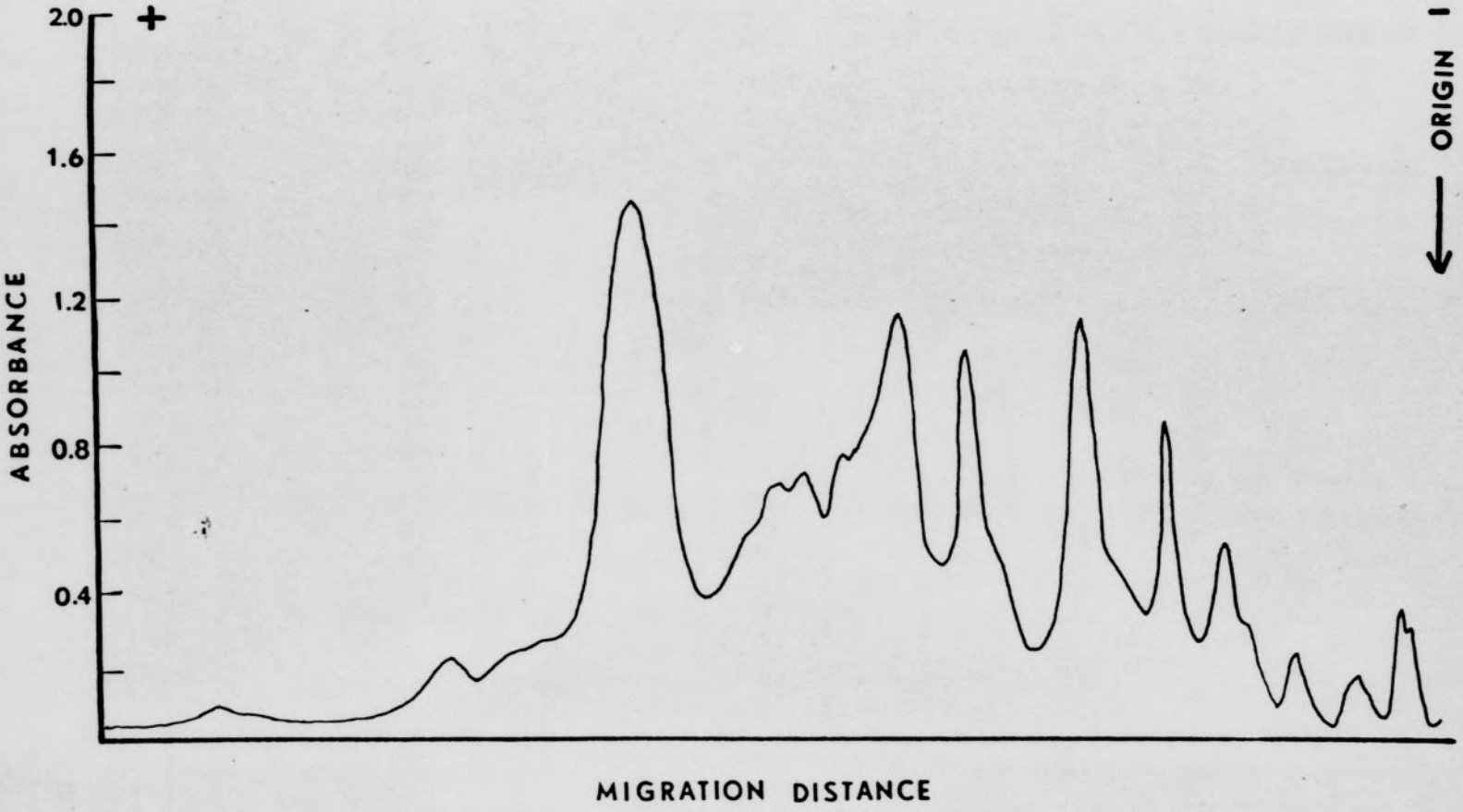
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Figure 8. Electrophoretic Separation of Glycerol + Galactose Heat Supernate, 42 Hours

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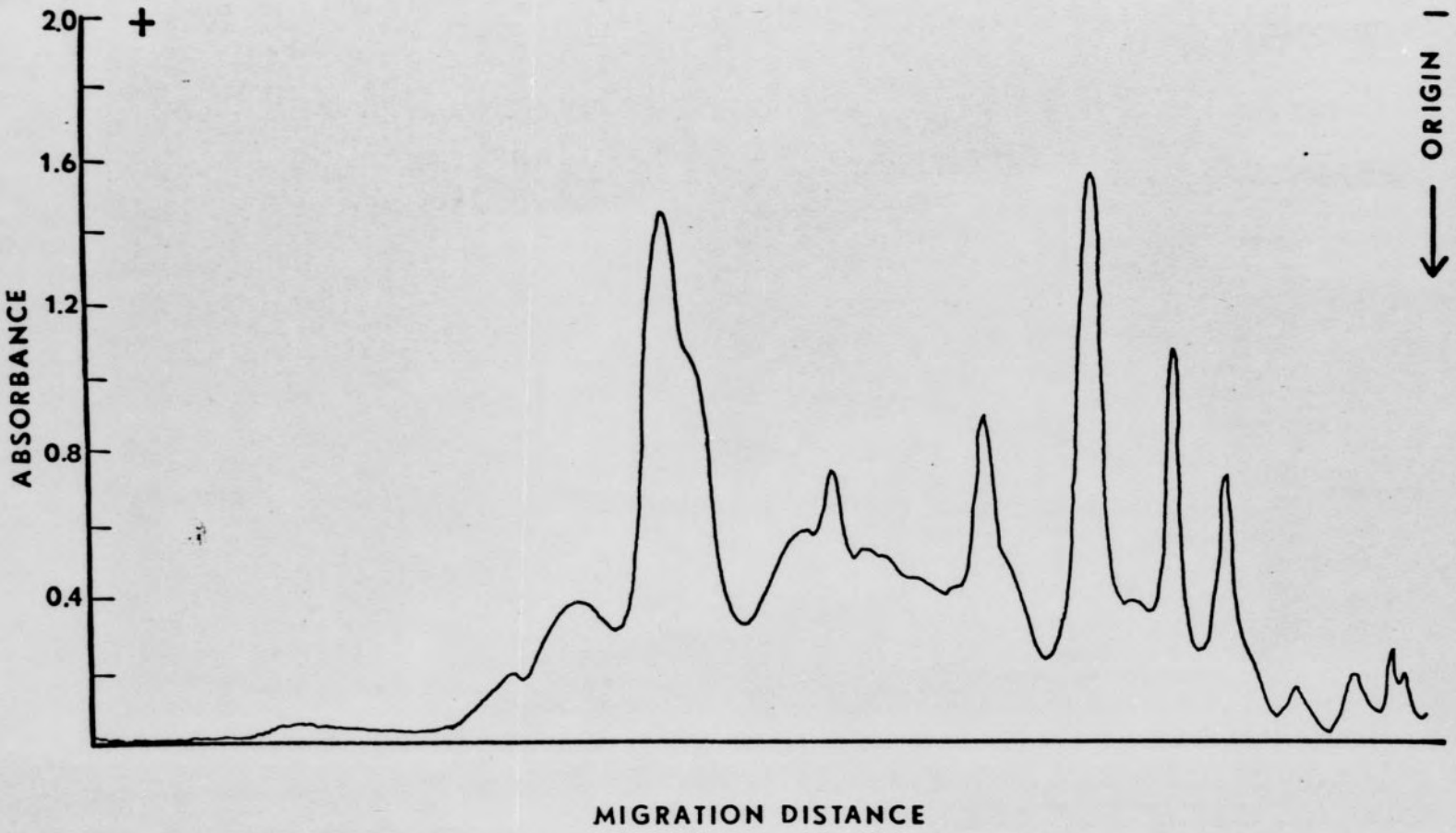
Figure 9. Electrophoretic Separation of Glycerol + Galactose Heat Supernate, 49 Hours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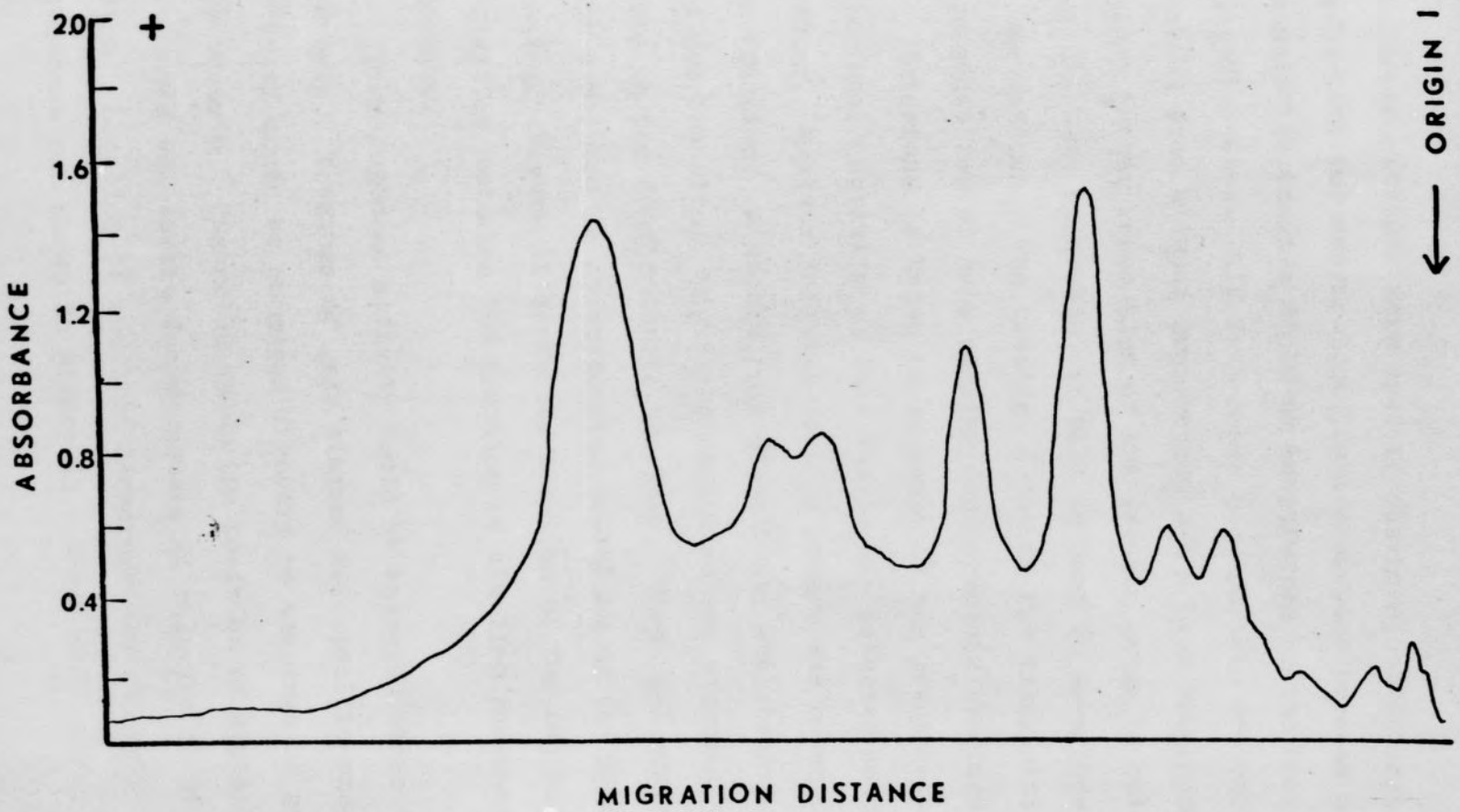
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Crude extracts were used to obtain electrophoretic separations for enzyme localization studies because any thermolabile enzymes would be inactivated by the heat precipitation step. Figure 3 shows a comparison of tracings obtained from a crude preparation and a heat supernate. Because better resolution of the protein peaks is obtained from the heat supernate, it will be used to show localization of the enzymes. The tracings selected for illustration are representative of many similar densitometric tracings.

Invertase is known to increase in the presence of galactose. Activity of this enzyme was detected as described earlier. Maximum activity of this enzyme was detected in the 4mm fraction. A second peak of activity was observed in the 9mm fraction. Migration distances are expanded 4.7 times in the densitometric tracings. These gel positions were converted to corresponding positions on the densitometric tracing. Figure 11 shows the location of the two invertase activities and also the position of alkaline phosphatase activity.

Dehydrogenase activity could be detected directly in the gels. Tracings of gels stained for specific enzyme activity could be compared directly to tracings of gels stained for protein. Figure 12 shows the position of glutamic dehydrogenase and lactic dehydrogenase in relation to the protein peaks. Location of succinic dehydrogenase and malic dehydrogenase are shown in Figure 13. The arrows on the

two figures indicate the location of visible bands of glutamic dehydrogenase and succinic dehydrogenase that were too light to be detected by the densitometer.

Figure 11. Electrophoretic Separation of Glycerol + Galactose Extract Showing Locations of Alkaline Phosphatase and Invertase Activities

A P Alkaline Phosphatase

I Invertase

——— Protein

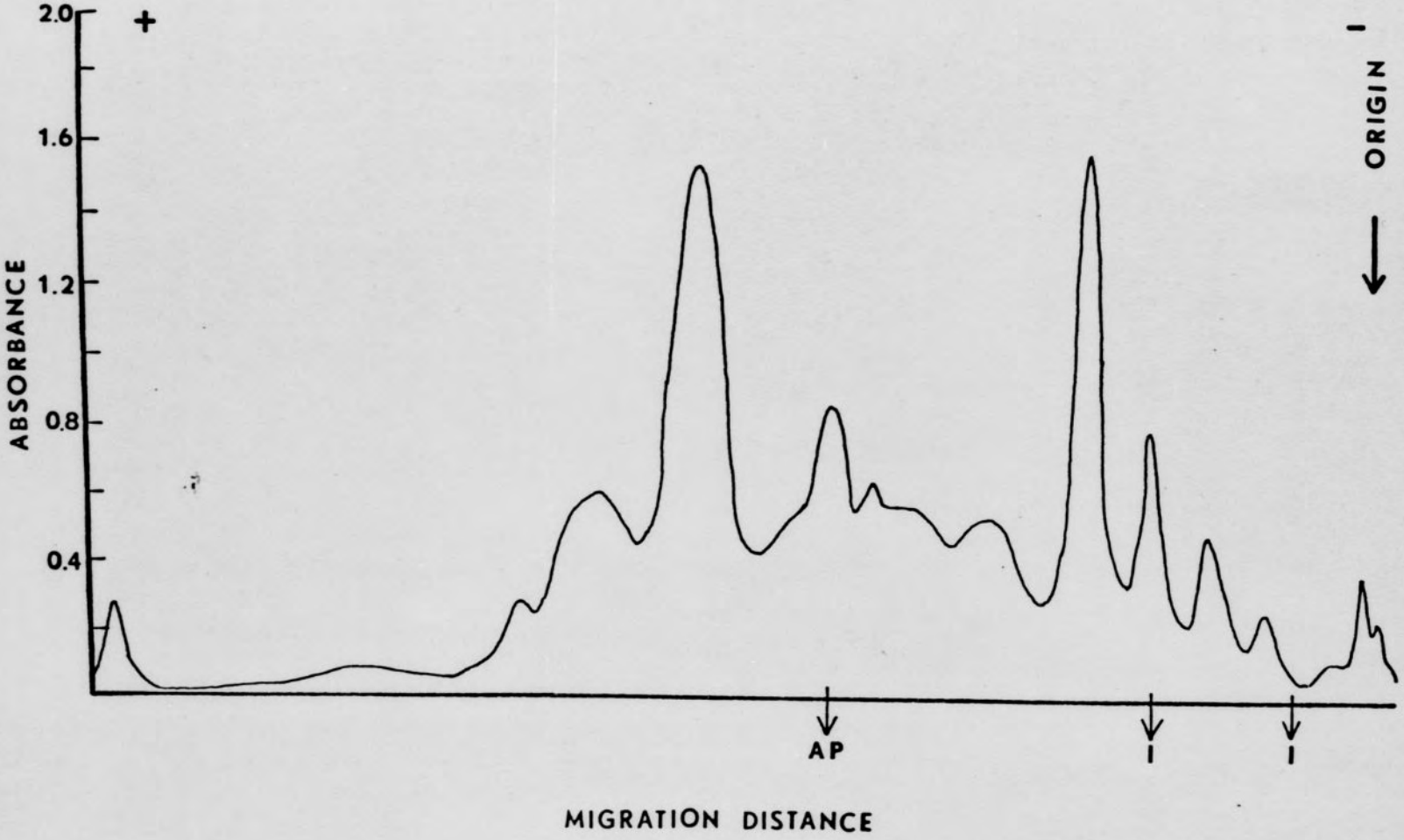
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Figure 12. Electrophoretic Separation of Glycerol + Galactose Extract Showing Locations of Lactic Dehydrogenase and Glutamic Dehydrogenase Activities

..... Lactic Dehydrogenase

----- Glutamic Dehydrogenase

——— Protein

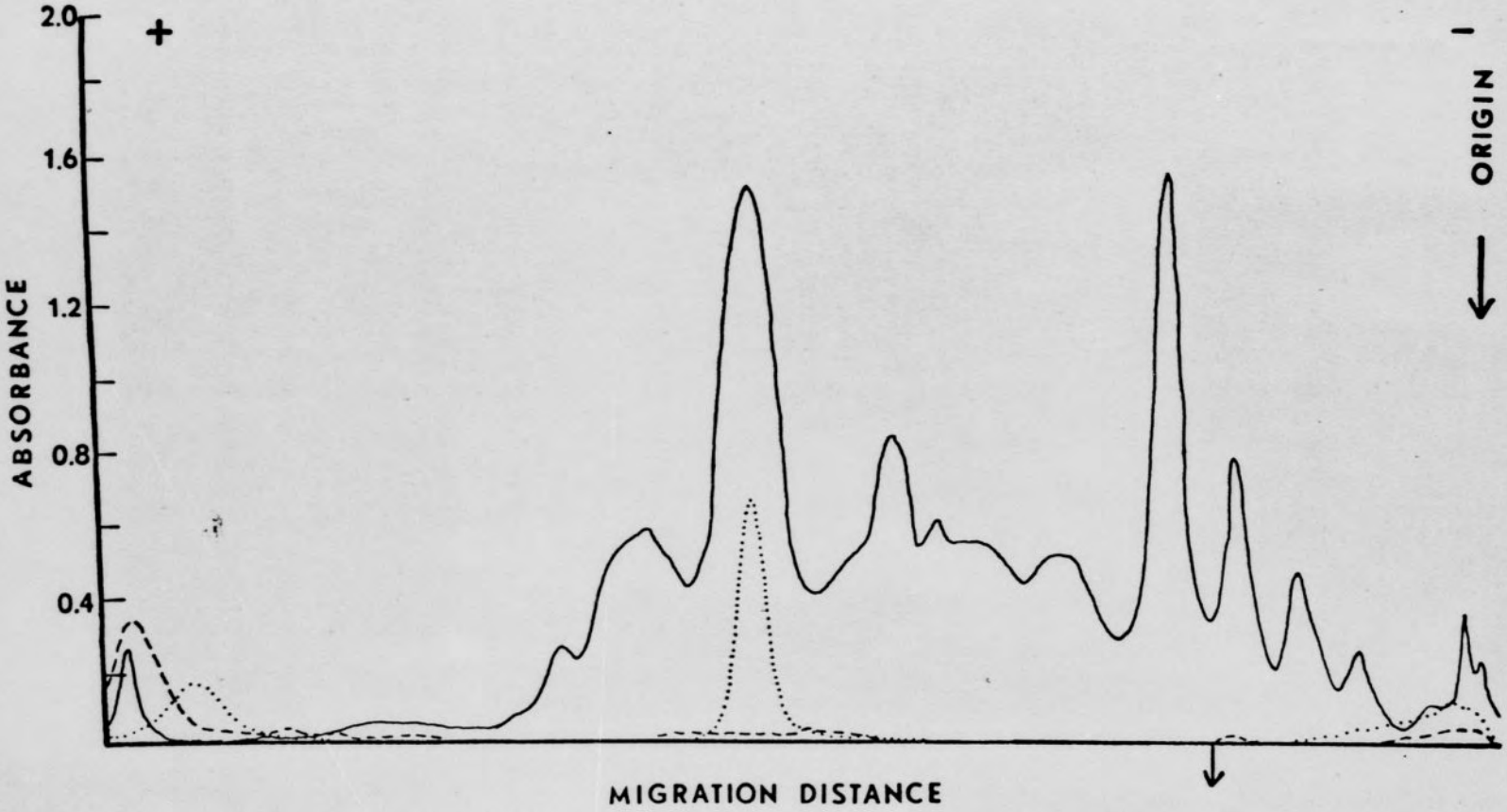
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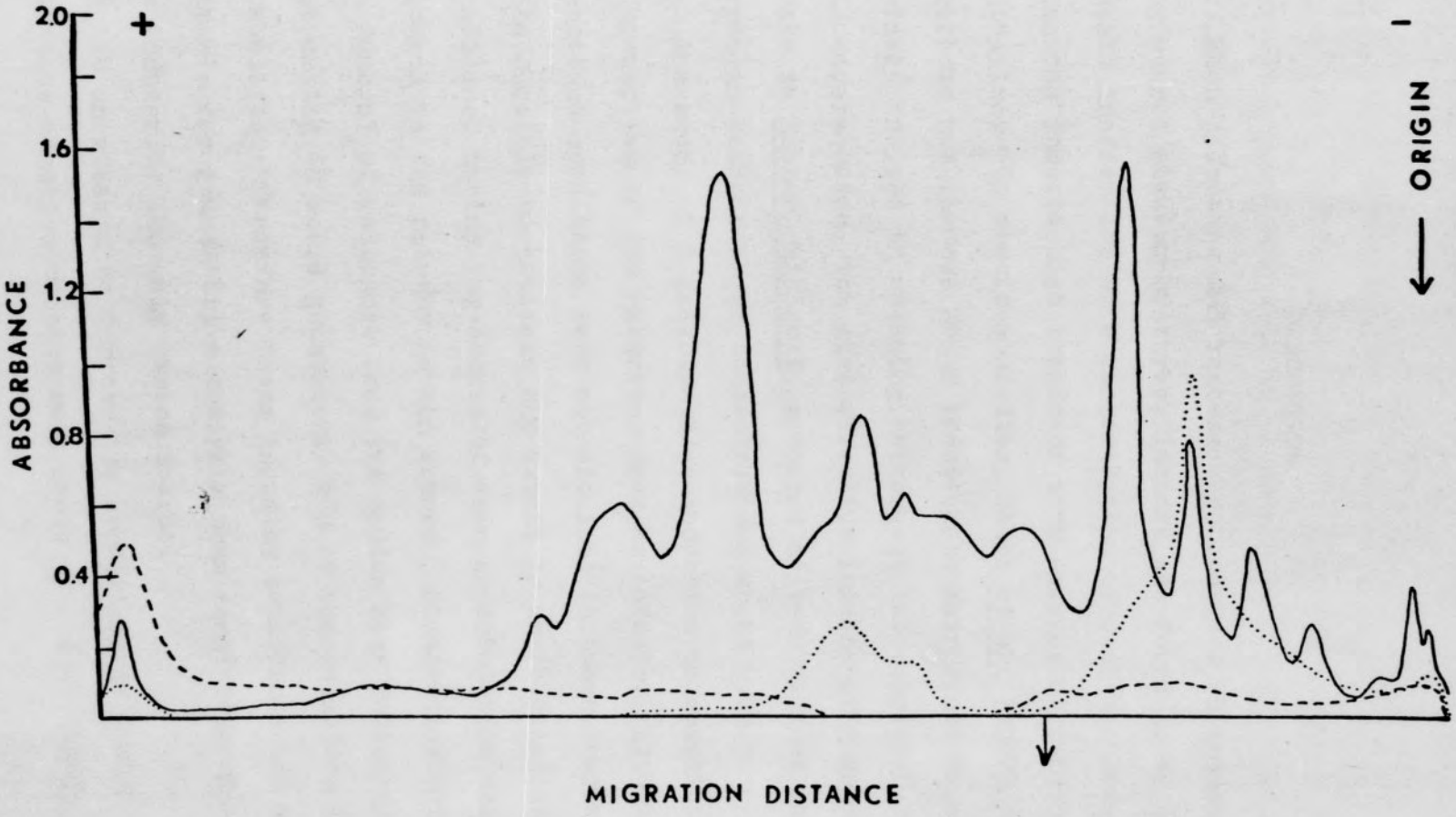
Figure 13. Electrophoretic Separation of Glycerol + Galactose Extract Showing Locations of Succinic Dehydrogenase and Malic Dehydrogenase Activities

----- Succinic Dehydrogenase

..... Malic Dehydrogenase

——— Protein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DISCUSSION

When galactose and lactose were compared as inducers in glycerol shaking cultures, lactose was found to be completely ineffective at a concentration of 0.05 M. However galactose induced high levels of both enzymes at this concentration. In previous studies, Bates et al. (1967) found galactose and lactose would induce both Neurospora β-galactosidase enzymes in standing cultures. Isopropyl-β-D-thiogalactopyranoside, the most effective inducer of β-galactosidase in Escherichia coli, produced no induction of this enzyme in Neurospora at concentrations up to 0.05 M.

The study of β-galactosidase induction is complicated by metabolism of the galactose used as inducer. Although gratuitous conditions were not attained in these studies, it is possible to estimate the extent to which galactose is metabolized during the course of each experiment in which glycerol is the primary carbon source. Studies involving the removal of galactose from the medium have indicated that during 48 hours growth only 25% of the galactose is metabolized. Therefore these inducing conditions are more suitable for quantitative analysis than conditions using the inducer as the only carbon source.

In an attempt to improve the conditions of induction galactose-negative mutants were isolated. Even though these

mutants were unable to grow on galactose as sole carbon source, they removed galactose from glycerol + galactose media to approximately the same extent as did the 411-L5-A strain. Therefore the use of these mutants for induction studies was abandoned.

Densitometric tracings of electrophoretic separations of heat supernates prepared from crude extracts of induced and noninduced Neurospora cultures show twelve major peaks. Each peak could represent a multifunctional aggregate or a population of proteins with the same electrophoretic mobilities or, less likely, a single protein. The occurrence of significant amounts of structural protein in Neurospora suggests that not all of the electrophoretic peaks are enzymes.

Variations in electrophoretic patterns observed with aging of the cultures indicated the necessity of following standardized conditions for growth and extraction of cultures in order to obtain reproducible patterns for comparison. When standardized growth conditions were followed, reproducible differences were observed in the electrophoretic patterns obtained from cultures grown under induced and noninduced conditions.

Because the pH 7.5 β-galactosidase enzyme is inactivated during electrophoresis, it could not be correlated with any specific protein band. The pH 4.2 enzyme does not migrate under the electrophoretic conditions used in these studies.

Activity could be detected only at the gel interface.

Certain other enzymes besides β-galactosidase are also known to increase in the presence of galactose. One of these, invertase, showed activity at two positions in agreement with results obtained by Eilers et al. (1964) and Metzenberg (1964). Metzenberg suggested that the faster migrating component was only an active subunit. The faster migrating component coincided with one of the peaks which increased after the addition of galactose.

The various other enzymes which were identified were randomly selected for their ease of detection to determine whether they migrated with the same electrophoretic mobilities or would be distributed among the different protein components. The results agree with those obtained by Strickland and Shields (1967) in the number of specific dehydrogenase bands detected. The localization of the enzymes showed they were widely distributed among the protein peaks revealed in the densitometric tracings.

The three peaks which increase after the addition of galactose cannot be accounted for in terms of known enzyme activities. One peak is due at least in part to invertase. One peak could be pH 7.5 β-galactosidase. The pH 4.2 enzyme is probably not represented in these electrophoretic separations because activity could be detected only at the gel interface. It has been determined that the pH 4.2 enzyme migrates and activity can be detected if the polarity is

reversed and a pH 5 buffer is used during electrophoresis (S. C. Hedman, personal communication). Therefore, unless both major increases are due to the pH 7.5 enzyme they must be due to proteins still not identified.

Since small amounts of galactose were metabolized, the increases could be due to the enzymes involved in galactose metabolism: galactokinase, phosphogalactose uridyl transferase, or UDP-galactose-4-epimerase. However, since an enzyme as unrelated as invertase is significantly affected by the addition of galactose, it is also possible that unidentified enzymes other than, or in addition to the enzymes involved in galactose metabolism, are affected.

The enzyme localization studies emphasize the point that one protein peak can represent more than one activity. Therefore, an increase in three protein peaks only indicates that a minimum of three enzymes are affected by the addition of galactose.

Although the mechanism of involvement of invertase remains unknown, these studies demonstrate that the effects of induction by galactose are observable by high resolution electrophoresis. This demonstration is significant because it allows a direct analysis of the gene-protein relationship without the uncertainties associated with studies of the gene-enzyme relationship based upon assays of enzyme activity. It is also apparent that alterations in amounts of certain proteins, and of electrophoretic migration characteristics

of some, accompany the process of induction in Neurospora. Such alterations are in contrast to the high degree of specificity of induction predicted by the Jacob-Monod model. These studies provide further evidence that, in at least this one eucaryotic organism, induction is controlled by a more complex mechanism instead of, or in addition to an operon-like system. Determination of the mechanism of this alternate or additional mechanism will require extensive studies of mutations of all structural genes whose expression is regulated by galactose.

SUMMARY

The isolation of a strain of Neurospora which shows improved growth characteristics on glycerol has allowed the development of a system in which galactose can be used as an inducer under conditions in which glycerol is preferentially utilized as the carbon source. Low levels of both β-galactosidase enzymes were produced when the organism was grown on glycerol alone. High levels of both enzymes are induced by the addition of galactose. A comparison of lactose and galactose showed lactose to be ineffective as an inducer at the concentration tested. Isopropyl-β-D-thiogalactopyranoside did not induce either β-galactosidase enzyme at the concentrations tested.

An attempt was made to improve the conditions of induction by isolating a strain of Neurospora which could not metabolize galactose but could be induced to synthesize β-galactosidase. However, the use of these mutants for induction studies was abandoned because the nature of the mutations could not be identified by the techniques available.

Preparations of soluble protein extracted from induced and noninduced Neurospora cultures were studied by high resolution electrophoresis to determine the effects of addition of galactose on Neurospora proteins. The protein extracts were subjected to disc electrophoresis on

polyacrylamide gels and the stained gels were quantitatively characterized on a recording densitometer.

When standardized conditions were followed for the growth and extraction of cultures, reproducible differences were observed in the electrophoretic patterns obtained from cultures grown under induced and noninduced conditions.

Enzyme localization studies demonstrated that various enzymes are widely distributed among the electrophoretic protein components and not concentrated in any one band. Beta-galactosidase could not be correlated with a specific protein band due to inactivation during electrophoresis. Invertase activity, also known to increase in the presence of galactose, was demonstrated in one of the protein peaks which increased after the addition of galactose.

Observed increases in three protein bands in response to addition of galactose suggest that at least an equal number of proteins are affected. The three peaks which increase cannot be accounted for in terms of known enzyme activities. Possible interpretations of these results are discussed.

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