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Title: The Effect of Inorganic Phosphate on the Production of Non-volatile, Aliphatic Organic Acids by Aspergillus wentii

Abstract approved: Richard P. Keeling

Inorganic phosphate has long been known to affect organic acid production and the types of acids produced are related to its concentration. In this study the effect of the variation of inorganic phosphate concentration on the production of non-volatile, aliphatic organic acids by a fungus was investigated. To find a suitable test organism 200 soil samples were taken and 306 acid producing specimens were isolated from them. Aspergillus wentii from these isolates was selected because of its ability to produce large amounts of several organic acids and its cultural characteristics. In A. wentii cultures having low phosphate concentrations (0.025 and 0.05 g phosphate/l) citric acid production was initiated first, while in the higher phosphate concentrations (0.5 and 1.0 g phosphate/l) malic acid was

produced first. Another effect of phosphate concentration was the retardation of weight accumulation in the cultures having low phosphate concentrations. An event not related to phosphate concentration was the decrease of disappearance of malic acid after growth became limited in a culture, which coincided with fumaric acid initiation.

THE EFFECT OF INORGANIC PHOSPHATE ON THE PRODUCTION OF NON-VOLATILE, ALIPHATIC ORGANIC ACIDS BY *Aspergillus wentii*

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R.H.K.

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INTRODUCTION

Secondary Metabolism and Cellular Differentiation

Secondary metabolites are chemically diverse substances catalyzed from primary metabolites by enzymes produced by microbial cells. These metabolites are produced when replication is limited or stopped or from processes which are intensified in limited or sub-optimal growth (Weinberg, 1974; Bu'lock, 1975). Primary metabolites are compounds such as amino acids, bases, lipids, and carbohydrates which are essential to replicating growth (Smith and Berry, 1974). As opposed to primary metabolites, secondary metabolites have no known function in the growth of the produced cell and most are biologically inert (Weinberg, 1974). Some have proved useful to man such as the antibiotics. However, secondary metabolites are more than just compounds of unusual chemical structure, such as the antibiotics. They can be unusually large accumulations of common cell components such as amino acids or citric acid cycle intermediates (Bu'lock, 1975). Several theories as to the function of secondary metabolism in an organism have been proposed. These are reviewed by Bu'lock (1975), Weinberg (1974), and Demain (1968).

Because secondary metabolites are diverse chemically, they can best be classified by a consideration of the

biosynthetic pathways which produce them and therefore link them to the comparatively uniform network of the primary biochemical processes (Smith and Berry, 1974). Smith and Berry (1974) have proposed several catagories according to their mode of synthesis. They are:

1. Acetyl Coenzyme A and fatty acids
2. Mevalonic acids
3. Amino acids
4. Sugars
5. Shikimate and/or the aromatic acids
6. Citric acid cycle intermediates
7. The products of several metabolic pathways (Smith and Berry, 1974)

Characteristically, the majority of secondary metabolites are produced by only one or a few species. They are genotypically specific. Also, they are phenotypically specific in that their production is extremely sensitive to present and past cultural conditions (Bu'Lock, 1975).

Secondary metabolism is an aspect of cellular differentiation (Bu'lock, 1975). Differentiation refers to the appearance of any new cellular property (a change in morphology or in chemical composition or activity) which makes that cell different from its parents or its previous self (Dyson, 1974). As stated before, secondary metabolism is initiated as a cell's replicating growth is limited or stopped and the cell begins synthesizing new enzymes. It is the opinion of Dyson (1974) that such induction and

repression of enzymes is a manifestation of cellular differentiation. The limitations of growth that are associated with the synthesis of secondary metabolites are partly or wholly external parameters such as the concentration of a particular nutrient. Other factors are pH, temperature, light, redox potential, oxygen tension, and osmotic pressure (Bu'lock, 1975; Berry, 1975; Weinberg, 1974). However, limiting replicative growth does not necessarily determine the pattern of secondary metabolism that is induced in a culture, but rather the particular type and the intensity of that limitation is determinative (Bu'lock, 1975).

Therefore, genetic and environmental factors must be considered in order to understand secondary metabolism (Bu'lock, 1975). In industry these factors have long been recognized and exploited both in the search for and the production of secondary metabolites.

Organic Acid Production and the Effect of Phosphate

As a group the fungi have proven to be particularly good producers of secondary metabolites and this ability has been utilized commercially (Bu'lock, 1975). One group of fungal metabolites that is commercially produced is the organic acids. In fact, their production was the beginning of the modern fermentation industry with the production of citric acid in 1923 (Miall, 1975).

Characterization of the effects of cultural conditions on the production of organic acids was a prerequisite

to their commercial production (i.e. Currie, 1917). To this end much time, effort and money has been invested to determine the optimal cultural conditions for their production. However, industrial mycologists typically search for conditions that will allow the maximum production of a single acid while minimizing other metabolites, particularly other acids (Weinberg, 1974). Also, they prefer organisms that are very active biochemically and produce large amounts of a single acid. An organism that produces one acid can permit the use of simpler purification processes with decreased production costs. This approach has consequently overlooked those species with less metabolic activity as well as those that simultaneously produce several organic acids. Also, since a single product is usually being sought, the range of metabolites that an organism produces is usually overlooked.

In regard to the range of metabolites that fungi produce, E. D. Weinberg (1974) has stated, "Secondary metabolites are formed typically as a family of closely related molecules and the proportions of the members of a family are inorganic phosphate dependent." The fact that phosphate affects secondary metabolism has been long known (i.e. Shu and Johnson, 1948; Foster, 1949; Karow and Waksman, 1947; Lilly and Barnett, 1951), but most of the effort has gone into manipulating the phosphate conditions to provide a single product rather than to determine how its variation would affect the range of secondary metabolites that an

organism might produce. In addition, the number of species that have been scrutinized in the investigation of the influence of phosphate concentration is small because of the previously mentioned search for organisms that might produce large amounts of specific metabolites.

The intent of this investigation is to add to the knowledge of the effect of the variation of phosphate on the production of different secondary metabolites. This was done by using a specimen that produces several non-volatile organic acids in reasonable large amounts, culturing it on media with various phosphate concentrations, and analyzing the acids produced. One prerequisite for the specimen was that it not be a species that has been intensively studied such as Aspergillus niger or Penicillium notatum. The specimen used was Aspergillus wentii. This yellow-brown species of Aspergillus is considered to be less biochemically active than either the black Aspergilli (A. niger group) or members of the Aspergillus flavus group (Raper and Fennell, 1973). However, some investigations of A. wentii have reported it as being potentially useful for citric acid production (Raper and Fennell, 1973).

MATERIALS AND METHODS

Isolation and Screening of Specimens

Two hundred soil samples were collected from Lyon, Brown, Riley, Pottawatomie, and Phillips counties in Kansas and from Grand Lake and Rocky Mountain National Park in Colorado. The isolation medium was Czapek's Solution Agar (Difco) modified to contain 15% sucrose (Good Value) and 125 ppm Combiotic (Pfizer) to suppress the growth of bacteria. Calcium carbonate was used as the acid indicator at a concentration of 0.2% (Foster, 1949). From each soil sample was taken 0.1 g of soil which was suspended in 10 ml of sterile distilled water. To petri dishes containing the selective medium was transferred 0.5 ml of this suspension and spread with a sterile, glass "hockey stick" (Foster, 1949). The formation of a clear zone under a colony in the otherwise insoluble calcium carbonate indicated that acid was being produced by that colony (Foster, 1949). The inoculated plates were incubated for 14 days at room temperature and inspected for acid producers periodically. By this method 306 acid producing fungal specimens were isolated.

Of the 306 isolates 268 were investigated for their ability to produce several non-volatile acids. The remaining isolates were of the Aspergillus niger group and were not considered because of the previously stated reasons. The

cultures were screened by culturing each isolate in a 16 X 125 mm screwcap culture tube containing 5 ml of Czapek's (Dox) Broth (Difco) modified to contain 15% sucrose. The tubes were incubated at 25°C for 7 days on a continuously rotating Wedco tube roller (15 rph). After incubation the medium was removed from the tubes and each sample was analyzed by thin-layer chromatography (Denison and Phares, 1952). Microcrystalline cellulose plates without flourescent indicator (20 x 20 cm Prekotes, Applied Science Laboratories) were spotted with 39 samples per plate using 0.5 to 0.9 mm I.D. by 75 mm capillary tubes (Scientific Products). The solvent system was ethyl ether:acetic acid:water (13:3:1). The solvent front was allowed to advance 12 to 15 cm on the plate. The acetic acid was removed by steam. The plate was heated on "dry phase" in an automatic autoclave to prevent condensation of water and then steamed in a manual autoclave. The manual autoclave was pressured to 14 psi, then de-pressurized to reduce condensation in the autoclave before the plate was inserted. The plate was placed in the manual autoclave with the door open (about 1 cm) immediately after removal from the automatic autoclave. The steam was adjusted until its flow could be heard well and the plate was steamed for 4 1/4 minutes. The positions of the acids were indicated by the color reaction of 0.4% brom cresol green in ethanol, adjusted with NaOH saturated ethanol to pH 5.4, when sprayed on the TLC plate. Any sample that produced an R_f value

different from citric acid was spotted again using a 5 ul applicator (Cordis). The results showed that 59 isolates produced acids other than citric acid. Of these 59 isolates 18 produced large amounts of 2 or more non-volatile acids.

The isolate that produced a large amount of mycelium in a relatively short period of time and produced large amounts of several acids was identified as Aspergillus wentii. Its cultural and morphological characteristics allowed it to be easily differentiated from contaminating organisms. Three different isolates of A. wentii were selected for further study. The isolate of A. wentii that grew better than the others and was used in the experiment was isolated from a soil sample taken from a wheat field 1/2 mile west of the end of the paved portion of South Prairie Street in Emporia.

Submerged Culture and Acid Analysis

Seed flasks of the A. wentii were prepared on 25 ml of Czapek's Solution Agar (Difco) in 125 ml Erlenmeyer flasks and incubated at 30°C. In four days conidia were abundant. For the inoculation of the submerged cultures, a suspension of conidia was prepared by adding 40 ml of 0.1% agar in water to a seed flask and gently brushing with a sterile paint-brush (Vezina and Singh, 1975). One ml of the conidial suspension was used to inoculate each of a series of 125 ml Erlenmeyer flasks containing 50 ml of one of the four phosphate media containing 0.025, 0.05, 0.5, or 1.0 g/l

of K_2HPO_4. All the media contained the following components in equivalent amounts: 3 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSo_4 \cdot 7H_2O$, and 150 g sucrose in one liter of distilled water. All cultures were incubated at 27-28°C on a rotary chest shaker (Lab-Line Instruments, 160 rpm). The glassware was cleaned by an Aqua Regia rinse (nitric acid: hydrochloric acid; 1:3), 3 distilled water rinses and one deionized water rinse. The dry weight of samples was determined by suction filtration through tared 4.25 cm Whatman no. 1 filter paper, dried overnight in an oven at 90°C and then placed in a vacuum dessicator over phosphorus pentoxide before weighing with a Mettler analytical balance. The pH of the filtered culture medium was determined with a single probe Mini-pH-Meter (Markeson).

To purify and concentrate the acids produced by the fungus a modification of the ion exchange method of VanEtten and McGrew (1957) was used. A 1.5 X 25 cm Econo Column (BioRad) was filled to a depth of 3 to 4 cm with Dowex 1 X 1 in the OH^- form. (Fig. 1) Eluant flow was facilitated by connecting the column to a vacuum manifold (Richards, 1975). Ten ml of the sample was added to the column and slowly drawn through by a slight vacuum. Two hundred and fifty ml of distilled water was eluted through the column at full vacuum to remove the neutral and basic medium components. Care should be exercised to remove the residual sucrose from the samples; otherwise, a green precipitate is

formed by its reaction with the HCl used to elute the sample acids from the column. After the other components of the medium were eluted about 3 ml of 1.0 N HCl was added to the column and about 30 ml of distilled water was used to elute the acidic components from the column. The eluant was collected in a 18 X 125 mm test tube. The acid sample was taken to dryness twice in a glass petri dish at 100°C to remove residual HCl. The sample was rehydrated in 2 ml of distilled water. This system was expanded to 10 columns to process samples quickly (Fig. 2).

The acids were identified by using the previously described TLC method and a solvent system developed by this investigator using ethyl ether:tert-butanol:formic acid: water (10:5:3:2) with 0.1 mm microcrystalline cellulose, 20 X 20 cm TLC plates (E. Merck, Darmstadt, Ger.). This solvent system gave better resolution and separated several acids that other solvent systems would not separate (i.e. citric acid from phosphoric acid and fumaric acid from itaconic acid). The resolution obtained with this solvent system and Prekotes was not good, necessitating the use of the Merck plates. Acid standards used for acid identification were of reagent grade.

Unfortunately, a good analytical method was not found to quantitate the acids of the sample or to remove contaminating medium components (NO_3^-, $PO_4^{=}$, $SO_4^{=}$) with the materials and equipment available. The results of this

problem was that relative concentrations of identified acids were based on spot size and density. The scale of density and size was as follows: 1--faint, 2--light, 3--moderate, 4--heavy, and 5--brightly heavy.

Figure 1. This closeup shows an ion exchange column with its reservior, column, sidearm flask, and vacuum attachments.

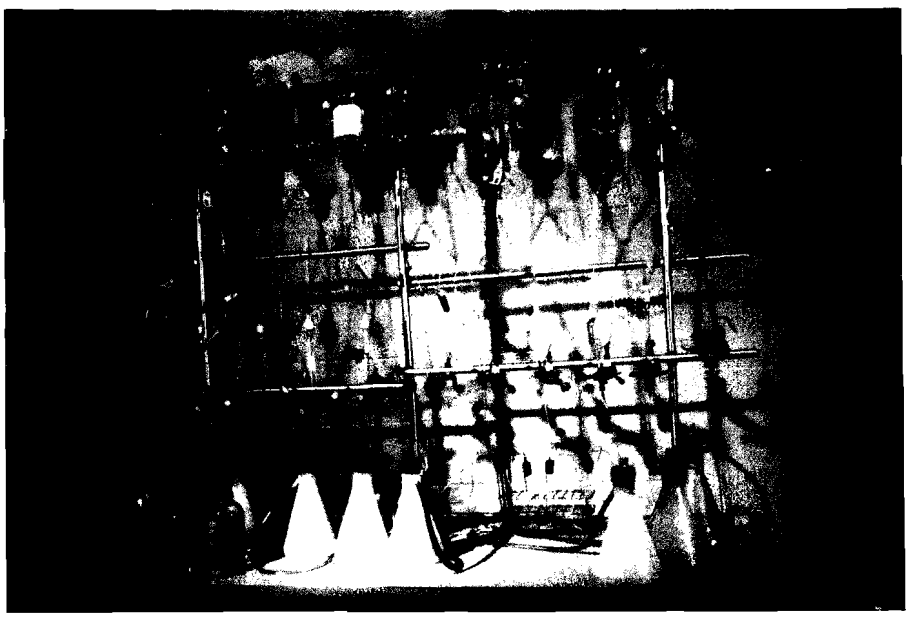

Figure 2. The 10 column ion exchange system was used to process the fermentation samples.

In cultures having low phosphate content (Fig. 3 & 4) the end of balanced growth occurs at the time when 30 to 40 mg of mycelium have accumulated. In higher concentration of phosphate (Fig. 5 & 6) the end of balanced growth does not occur until about 45 to 50 mg of mycelium have accumulated. In the study of the TLC plates (data not presented graphically of the cultures having lower levels of phosphate (0.025 and 0.05 g phosphate/l) samples, phosphoric acid was not detected during the course of the investigation, while on those TLC plates of the samples of the higher phosphate concentration (0.5 and 1.0 g phosphate/l) phosphoric acid was detected on the TLC plates during the entire fermentation.

Phosphate concentration influenced the fermentation by providing a buffering effect at the outset as well during the course of the fermentation. The initial pH of the media ranged from 7.3 to 5.0 (Fig. 3, 4, 5, & 6) which corresponded to the amount of phosphate added. In all cases the pH decreased rapidly for the first 48 hours and then a plateau was reached between pH 3.1 and pH 2.5. The final concentration of total acid (in pH) was dependent on phosphate concentration. Also, when compared with the growth curve the pH plateau is reached about 12 hours before balanced growth ends and does not change more than 0.5 pH after that.

Organic Acid Production

The level of phosphate contained in a medium influenced the initiation of the production of the various acids in A. wentii. In the cultures containing 0.025 and 0.05 g phosphate/l (Fig. 3 & 4) citric acid was initiated at least 12 hours before malic acid. In the other cultures (Fig. 5 & 6) malic acid appeared first. In no culture did the 2 acids appear at the same time. Also, the amount of phosphate may be responsible for the unusual time of initiation of malic acid in the culture containing 0.05 g phosphate/l (Fig. 4). The start of the production of malic acid was delayed until 60 to 72 hours which coincides with the end of balanced growth. Thus, at this level of phosphate, the initiation of malic acid production is 12 hours later than at any other concentration of phosphate.

Several aspects of the production of the organic acids unrelated to phosphate were also observed. In all cultures malic acid reached a peak concentration between 60 and 84 hours and then decreased or disappeared by 96 hours. The accumulation of malic acid was associated with balanced growth while its disappearance was associated with the storage phase. Citric acid generally accumulated in all cultures following the initiation with an increase in the rate of its accumulation during the storage phase. The appearance of fumaric acid production was associated with the storage phase and the decline of the malic acid concentration. Fumaric

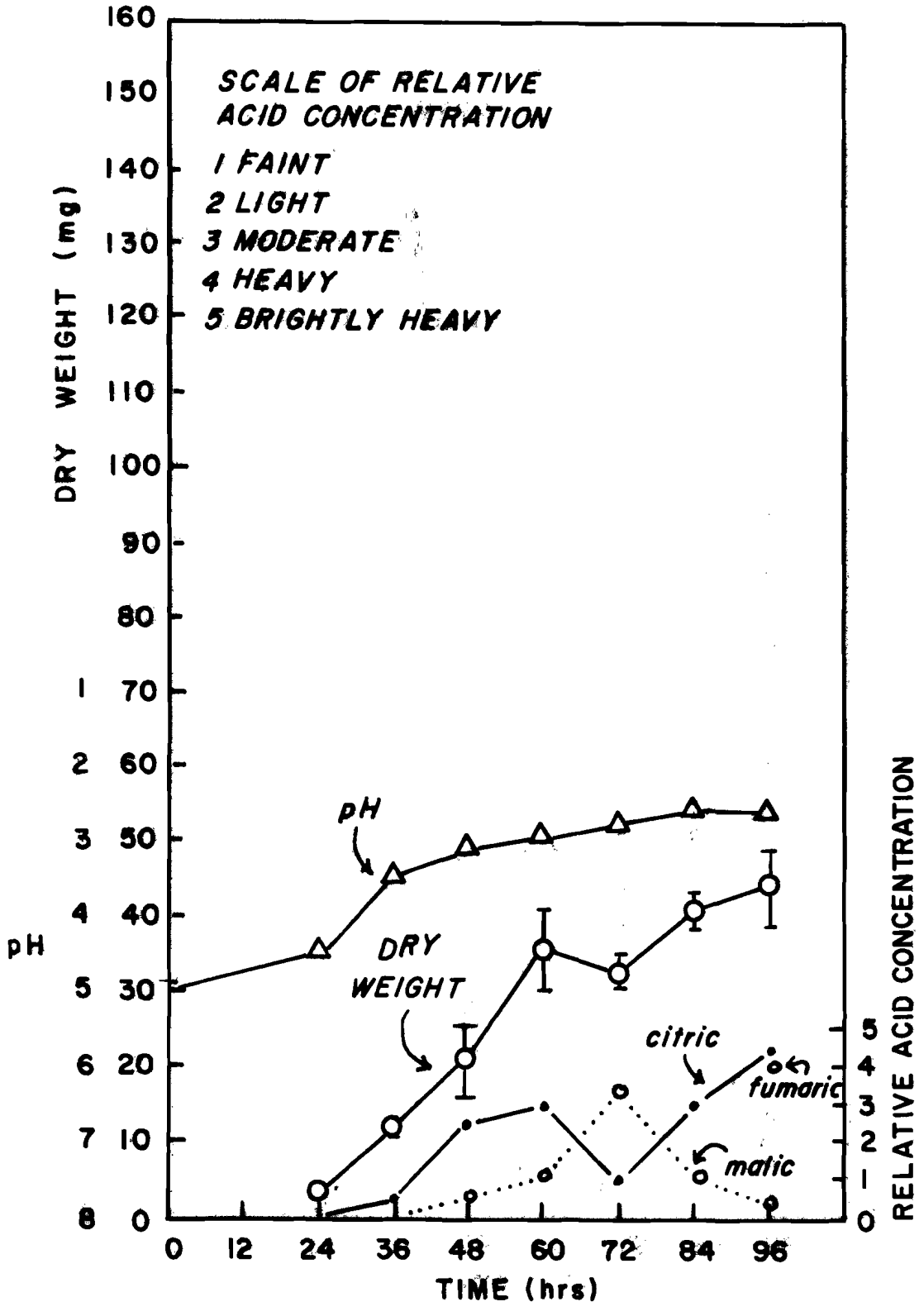

Figure 3. Dry weight, pH and Relative Acid Concentration in the 0.025 g phosphate/l culture

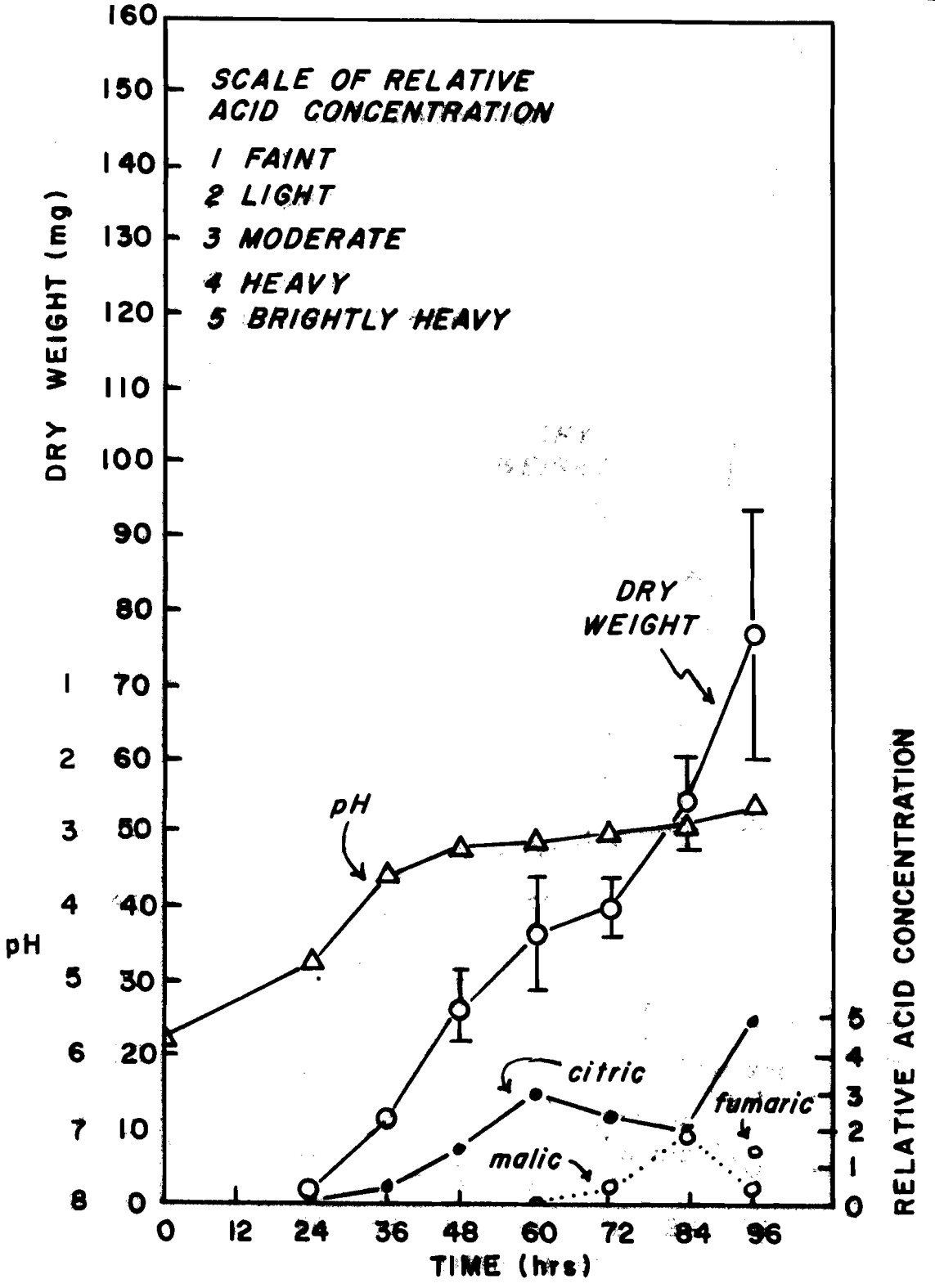

Figure 4. Dry weight, pH and Relative Acid Concentration in the 0.05 g phosphate/l culture.

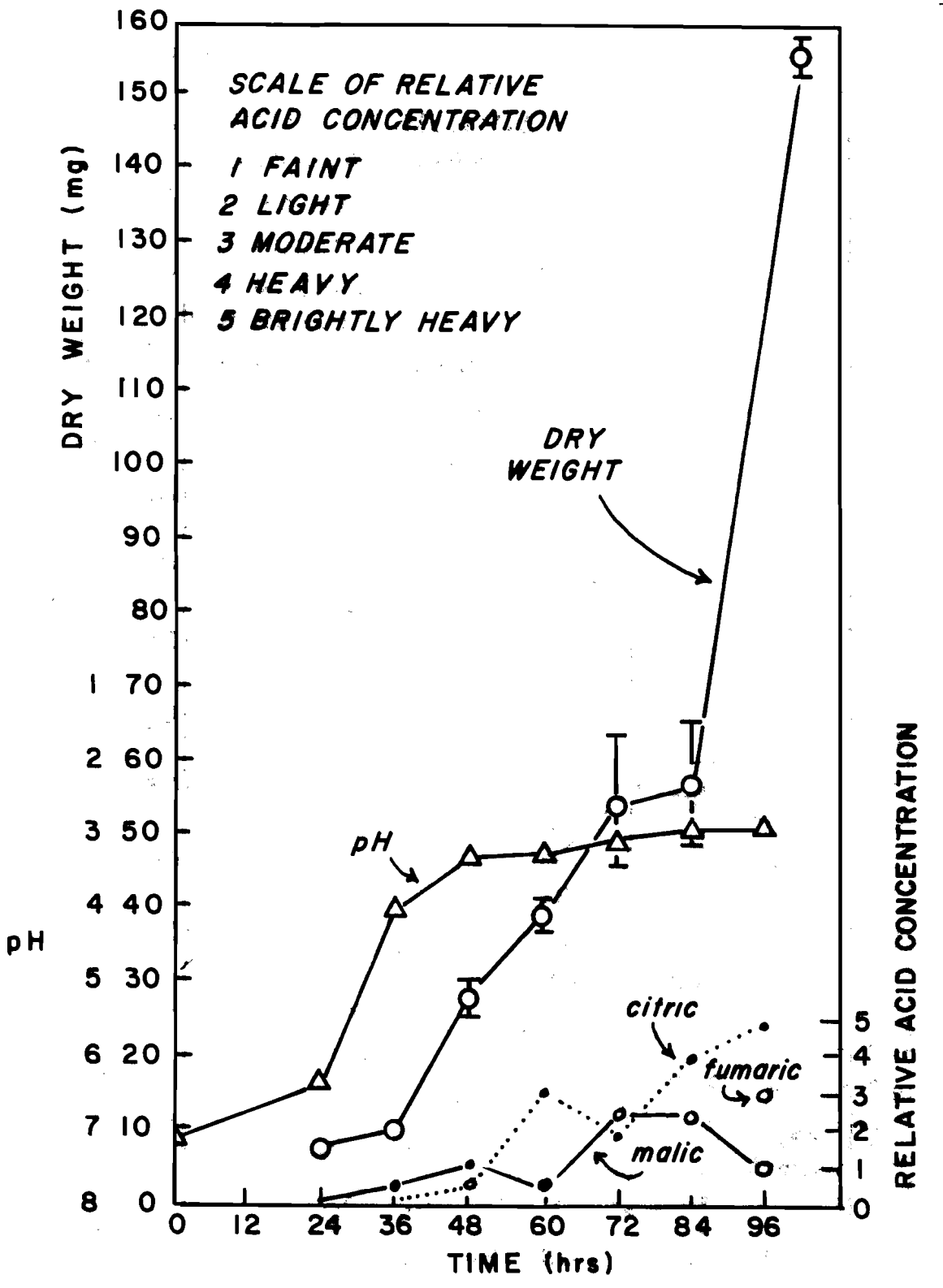

Figure 5. Dry weight, pH and Relative Acid Concentration in the 0.5 g phosphate/1 culture.

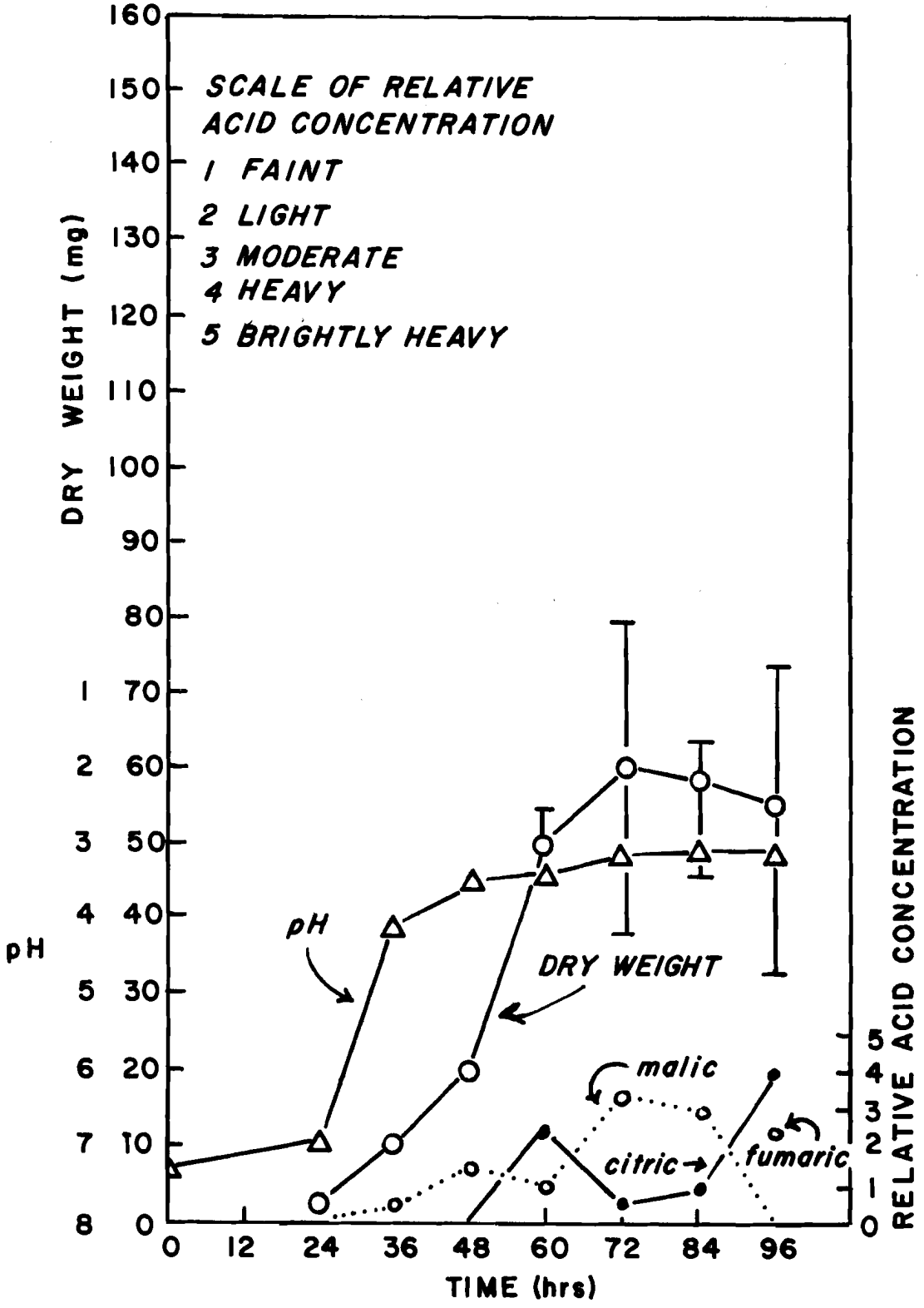

Figure 6. Dry weight, pH and Relative Acid Concentration in the 1.0 g phosphate/l culture.

acid only appeared at 96 hours and was not seen before that time. Its high concentration indicates that it was being very actively produced at that time.

The affect of the variation of the phosphate concentration on the initiation of organic acid synthesis is the opposite of that reported by S. M. Martin and R. Steel (1955) in their work with Aspergillus niger. In their study the addition of phosphate caused citric acid to be produced before malic acid. In this investigation A. wentii produced malic acid first when phosphate was added. It would appear that low phosphate concentrations facilitate the start of the production of citric acid, while the higher phosphate concentration cause the initiation of malic acid. However, the effects Martin and Steel reported "appeared to be true phosphate effects rather than pH effects, since the addition of phosphate did not alter the shape of the pH curves or the final pH (Martin and Steel, 1955)." In this study the same can be said as the pH changes were rapid, they did not correlate with the other activities that were measured, and the final pH of the cultures were not much different.

Other experiments were attempted with phosphate concentrations up to 30 g/l, but were not reported here. Changes in pH indicated that acid was being produced in the cultures, but increasing the phosphate concentration beyond 3 to 5 g/l resulted in phosphate ion interference that made the TLC assay impossible using the Denison and Phares (1952)

solvent system. The acids could not be separated. Furthermore, the capacity of the ion exchange resin (in the amount of resin used in this study) was over reached with the high level of anion. However, further study of these higher phosphate concentrations using the solvent system developed by this investigator might be able to separate the acids, thus allowing their identification. Increasing the amount of ion exchange resin in the columns would also help because of the high amount of phosphate anion.

In the higher phosphate study when phosphate concentrations were increased above 15 g/l, growth started to be inhibited in the cultures until at 30 g/l no growth occurred in the culture medium. These results along with those from the low phosphate cultures show that the phosphate concentrations between 0.05 g and 15 g/l permit maximum growth which was predicted by Weinberg (1974).

Other effects unrelated to phosphate concentration were observed. Malic acid and citric acid are produced in excess during the balanced growth phase. Therefore, they are unusual cases of metabolism. A few reports of secondary metabolites being produced during balanced growth have been reviewed by Bu'lock (1975). In the work of Martin and Steel (1955) citric acid and malic acid accumulated during the balanced growth phase although the concentration of citric acid greatly increased after balanced growth ended and malic acid remained at low levels. The decrease or disappearance

of malic acid from the medium after balanced growth ends conflicts with the generally accepted definitions of secondary metabolism as stated before. Thus, malic acid in Aspergillus wentii must be considered a special case with reference to secondary metabolism. It is the opinion of this investigator that malic acid is a secondary metabolite, since it does meet most of the criteria of a secondary metabolite as defined by Bu'lock (1975).

Any attempt to explain the disappearance of the malic acid from the medium would require the consideration of several interconnected factors. One reason may be related to the accumulation of citric acid and fumaric acid. During cellular regulation of the citric acid cycle (Lehninger, 1975), citric acid might become more concentrated if enzymes that produce it are more active or more abundant or its precursors are processed more efficiently. If this be the case, malic acid would be processed to oxalacetic acid more readily. Fumaric acid accumulation might mean that the enzymes catalyzing the synthesis of malic acid are limited in some way or that the enzymes producing fumaric acid are more active or abundant. Because fumaric acid appears late in the fermentation it is a typical example of a secondary metabolite as defined before.

In a consideration of the unexpected deceleration in the growth rate following the balanced growth phase and just prior to the storage phase, one may postulate that a critical

nutrient has been exhausted, thus arresting growth (Bullock, 1975). With the end of replication new enzymes are produced for the synthesis of storage products, but do not add much weight to the mycelium. This would explain the deceleration, since little additional mass can be added to the cell until the new enzymes are synthesized.

As to what caused the end of balanced growth in the cultures, the results indicate that the exhaustion of phosphate in the cultures having low phosphate levels ended balanced growth and that the exhaustion of some other nutrient, perhaps nitrogen, arrested it in the high phosphate cultures.

Since all these activities--citric and fumaric acid accumulation, the unusual production and decrease of malic acid, nutrient exhaustion and the deceleration in mass accumulation--are associated with the period of time when balanced growth slows, it would seem that A. wentii has the same traits as other organism when it produces secondary metabolites. Although it grows somewhat slower than other organisms (Martin and Steel, 1955), its pattern of secondary metabolism is essentially the same as other filamentous fungi as described by Bu'lock (1975).

In the further study of the growth and acid production of A. wentii several areas need more investigation. A method needs to be developed to quantitate the acids produced such as the UV technique developed by Richards (1975).

Replication growth could be better monitored by using [illegible] assay for the nucleic acids or by using one of the methods suggested by Bu'lock (1975) rather than dry weight which has limitations when used with the fungi. The storage phase of the organism could be examined by using assays for carbohydrates and lipids. Finally, a longer fermentation period covering a wider range of phosphate concentrations is also needed.

In summary, this study has shown that phosphate does affect the organic acid production of A. wentii. In addition, it has given some knowledge of the growth characteristics of the organism on synthetic media.

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