CHAPTER - VIII

GENERAL DISCUSSION

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Very significant break throughs have been made by various groups of scientists during the last decade in understanding the metabolic functions of biotin. Whenever possible parallel studies were conducted to investigate the participation of biotin in animal and microbial systems. Thus it is now possible to delineate the universal and very specific roles of biotin in certain metabolic reactions.

In the present case biochemical changes associated with biotin deficiency have an important bearing on the growth of A. nidulans. In contrast to the results reported in other microbial systems, which showed the inhibition of growth by biotin deficiency Winzler[1]; Mistry and Dakshinamurti[2]; Ahmed and Rose[3]. Rao and Modi[4] demonstrated the increase in cellular synthesis due to biotin deficiency in A. nidulans. Such change in biotin deficiency was found to be associated with the increase in protein content, with concomitant decrease in lipid content of the mold. A relationship has been shown to exist between biotin status of the culture and the cellular permeability as evidenced by change in uptake rate of various nutrients like glucose, ammonia, and phosphorous by Desai and Modi[5]. The difference in the

growth between the normal and deficient culture was significant when ammonium nitrate was used as the nitrogen source. There was observed a 2 fold increase in the growth of the deficient culture. The growth curve of A. nidulans with respect to biotin deficiency showed that both normal and biotin deficient cultures reached optimum growth by 5 days of incubation. Biotin deficiency causes increase in the growth rate of A. nidulans without altering the total period required to complete the growth cycle.

Biotin deficiency is also resulting in a quantative difference in the total lipid content of the mold. A concomitant fall (14.8%) in the lipid was observed in the deficient culture as compared to the normal (Chapter III), at the same time there was an increase in the total intracellular protein.

The qualitative analysis of the proteins by gel electrophoresis showed the presence of additional protein bands in the deficient culture extracts. Qualitative and quantitative changes in the protein have been reported by various investigators during morphogenesis, ~~for~~ e.g. by Chet et al[6]; Kornberg et al[7]; and Jokusch et al[8]. When ammonium nitrate was replaced by casamino acids, there was an increase in the overall growth of the deficient culture as compared with the normal. With different amino acids as the sole nitrogen

source,it was found that the alipphatic group of amino acids i.e. alanine, glycine, serine and threonine showed a remarkable increase in growth of deficient culture as compared to the normal one.

With the increase in the protein synthesis of the deficient culture there was an increase in the cell pool concentration of glutamate, aspartate, serine and alanine. These amino acids were found to be formed in the initial stages of growth. The bound amino acids were higher than the free amino acids in the intracellular pool during the initial phase of growth i.e. till the 3rd day indicating a rapid conversion of free to bound amino acids, thereby leading to an increase in protein synthesis of deficient cells. The glucose grown cultures when transferred to media containing other carbon sources after 24 hours of growth showed succinate to be by far the best carbon source, the poorer sources being acetate, citrate and to some extent glycerol. The difference in the growth of A. nidulans on different carbon sources could be due to the regulation of key catabolic enzymes or of specific uptake systems as shown by Romans[9] and Kornberg[9,10].

When potassium nitrate was used as the sole nitrogen source the normal culture behaved like the deficient with

respect to the growth indicating that the repression effected by the ammonium ions when grown on ammonium nitrate was lifted. Data obtained from Chapter III suggests that change in the permeability properties of the cell due to the biotin deficiency may be due to alteration in the composition of the cell membrane especially in the content of proteins as shown earlier and decrease in the total lipid. Change in the permeability due to biotin deficiency has been shown in many bacteria by Shiio et al[11-13]; and Otsuka et al[14,15]; and in fungi by Katsuki[16,17], and Rao and Modi[4].

It was further observed that there was an increase in the mycelial mass of Aspergillus nidulans, on addition of either untreated or heated egg-white or avidin, which appears to be due to the breakdown of the protein by proteases. Earlier studies had revealed that the addition of avidin to the cultures of A. nidulans, resulted in the decrease of fatty acid with resultant increase in the cellular synthesis, when the fungus was grown on ammonium nitrate as sole nitrogen source. Further supplementing the medium with heated egg-white and raw egg-white, there was an increase in the total cellular mass. The growth kinetics of A. nidulans on different days of incubation using varying concentrations of egg-white showed that with an increasing concentration of eggwhite there was a gradual increase in the

total cellular mass of the culture, the best results were achieved when the optimum concentration of raw eggwhite, a heat-inactivated egg white was added. The total lipid on different days of growth under different culture conditions (Chapter IV) showed a steady phase of lipid content throughout the incubation period in the normal culture. In the culture grown in the presence of avidin there was a decline in the lipid content as the time of incubation increased. The supplementation of equal concentration of bovine serum albumin showed that it could act as an ideal substitute protein in place of eggwhite. The data also shows a marked decrease in extracellular ammonia from culture filtrates of heat inactivated eggwhite treated cultures and deficient cultures as compared to the control. There was also observed an induction in the proteolytic activity though the extracellular protein concentration did not vary significantly. There was also observed an induction in the proteolytic activity. The neutral pH 7.2 protease was not active in the control and heat-inactivated cultures (HEW) but showed maximum activity by the 3rd day of incubation in deficient cultures. The acid protease on the other hand was active only in the beginning phase of incubation but later on showed a decline. The results of Chapter IV are indicative of the fact that increase in

cellular synthesis on the addition of either untreated or heat-treated eggwhite to the cultures is probably due to the breakdown products which could be used as a readymade source of nitrogen. This can bring about an enhancement in the cellular mass of the culture. The high proteolytic activity during the initial stages of incubation would make the medium of egg-white added cultures highly nutritious for the mold, and then the mold at the same time can utilize more ammonia. These nitrogenous sources can then be converted into cellular protein via the ammonia assimilating route, i.e. NADP-glutamate dehydrogenase (Chapter 6) It becomes clear that the enhancement in total cellular mass was due to the rich source of protein present, in the eggwhite and permeability change brought about by the changed fatty acid ~~pertten~~ pattern in the membranes of A. nidulans in the presence of avidin.

The ammonia assimilation studies under different culture conditions showed that in HEW cultures where earlier there was a 2-3 fold increase in the total cellular mass over both the control and deficient cultures also showed an enhanced ammonia assimilation in these cultures. There was a 100% reduction in the extra-cellular levels of ammonia between the 3rd to 5th day of incubation refer (Chapter 5) in the case of HEW cultures as compared to the control. This

fall in the level of extracellular ammonia was most significant by the 5th day of incubation. The levels of intracellular protein on the other hand were the lowest in normal cultures and higher in Deficient and HEW cultures by the end of incubation phase. When the modified nitrogen sources like casamino acids were substituted in place of ammonium nitrate as reported earlier as sole nitrogen source, there was an increase in the mycelial mass of deficient cultures. A. nidulans in common with most fungi can grown on media containing only inorganic nitrogen such as nitrate or ammonium. Under these conditions all the essential organic nitrogen substances are synthesized and the relevant biosynthetic pathways in A. nidulans are probably closely parallel to those found in Neurospora crassa by Esser and Kuenen[18] and Kinghorn and Pateman[19]. Preliminary data also indicated that there was an enhancement in the total cell dry weight on supplementation of the medium with amino acids singly or in pools (refer Chapter 5). Conditions for the uptake of the supplemented amino acids were optimised and in case of serine there was an enhancement in the uptake in the initial five minutes of incubation with the HEW culture cells whereas no uptake was observed with normal and deficient cultures during the same time interval. Some intracellular changes in terms of the amino acid concentration

were also evident on incubation of the cells with serine. It would be of interest to note the fate of serine once it enters the cell, and to correlate these changes with the changes in the internal amino acid pool. The results indicative of an increase in the ammonia uptake in A. nidulans with the deficient culture and HEW culture, which further led to an increase in the total intracellular protein levels as compared to the control. As the major route of ammonia assimilation in A. nidulans is via NADP-glutamate dehydrogenase, this could be one of the factors affecting ammonia assimilation. There is also an increase in one of isoenzyme levels of NADP-glutamate dehydrogenase, (refer Chapter 6) which plays a role in enhancing the activity of this enzyme in deficient cultures. NADP-dependant glutamate dehydrogenase could be one of the factors, leading to high ammonia assimilation by micro-organisms[20-23], a similar role is detected in deficient cultures of A. nidulans.

In the earlier part it has been reported that there is an increase in the activity of $NADP^+$glutamate dehydrogenase during biotin deficiency, this can be correlated to the biosynthetic demands of the culture, which would be higher in the biotin deficient culture as compared to that in the normal culture. The results are in agreement with those of Hubbard and Hall[24] and Shiio et al[25], who observed

the enhancement in glutamate dehydrogenase activity during biotin deficiency in Bacillus cereus and Brevibacterium flavum, respectively. Recently it was demonstrated that $NADP^+$GDH is the major route of ammonia assimilation in Aspergillus nidulans[26]. At the same time it was reported that the ratio of NADPH/NADP, was found to increase from 1.2 to 2.2 during biotin deficiency[5]. This indicates that dehydrogenation reactions are more active in biotin deficiency to provide more reducing power needed for the cellular synthesis. The kinetic studies of $NADP^+$-dependent glutamate dehydrogenase are also indicative of the enhanced activity of this enzyme in deficient cultures as compared to the normal.

The results in the present investigation suggest that there is an enhancement in the activity of the proteases in the deficient and HEW cultures. This is accompanied by an increase in the total cell mass and protein content of the corresponding cultures. At the same time some intracellular protease production is also observed (refer Chapter 7). Our data allows us to support the results of Drucker[27], who finds that in Neurospora crassa protease release requires an inductive interaction between exegenous protein, homologus or heterologus protease and intact mycelium as well as carbon starvation. Whereas results of Cohen[28] establish the occurance of ammonium repression by proteases, but does not exclude the possibility of induction by exogenous protein.

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The Following were the list of papers presented at AMI Conferences from 1976 - 1979.

1. "Some Aspects of metabolic alterations during biotin deficiency in Aspergillus nidulans", presented at the 17th Annual Conference of Association of Microbiologists of India at Manipal from 13th to 15th December,1976.

2. "Factors affecting Ammonia assimilation in Aspergillus nidulans", presented at the 18th Annual Conference of Association of Microbiologists of India at Madurai from 21st to 23rd Dec.,1977.

3. "Induction of Extracellular proteases by egg-white in Aspergillus nidulans", presented at 19th Annual Conference of Association of Microbiologists of India at Baroda from 9th to 11th November, 1978.

4. "Production of Intracellular proteases by egg-white in Aspergillus nidulans", presented at 20th Annual Conference of Association of Microbiologists of India at Hissar from 1st to 3rd November, 1979.

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Effect of Egg-white & Avidin on the Growth of *Aspergillus nidulans* & on Its Proteases & NADP-GDH

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Increase in mycelial mass of *A. nidulans* on addition of either untreated or heated egg white or avidin appears to be due to the breakdown of proteins by proteases because of which additional source of nitrogen is made available. These changes were related to increased uptake of ammonia and greater levels of NADP-glutamate dehydrogenase. It is suggested that the enhancement in the total cellular mass is due to the availability of protein in the egg white and change in the fatty acid pattern of the membranes due to avidin affecting permeability.

THE role of biotin in cellular metabolism, particularly in carboxylation reactions and in fatty acid synthesis in both prokaryotes and eukaryotes is well known[1-4].

One discovery that led to our understanding of the involvement of biotin in enzymatic reactions was the isolation of avidin, the egg-white protein that binds biotin at specifically very low, micromolar concentration. Animals that were fed raw egg-white showed symptoms of biotin deficiency and loss of body weight[5]. The biotin deficiency was found to affect also the protein synthesis and mitochondrial oxidative phospharylation of the animal[6,7]. However similar studies carried out with the fungus *Aspergillus nidulans* showed that during biotin deficiency there was an increase in the total cell mass and it was suggested that this could be due to change in the permeability of the cells leading to higher uptake of nutrients[8]. We have now carried out detailed studies with active and inactivated avidin, and with raw and heated egg-white, and report that the enhancement of cell mass in *A. nidulans* under biotin deficiency or in presence of egg-white is a result of availability of better nitrogen/carbon monomers released on degradation of exogenous protein substrates by proteases which are induced by them.

Materials and Methods

A strain of *Aspergillus nidulans* obtained from the division of Mycology and Plant Pathology IARI New Delhi was used. Stock cultures were maintained by weekly subculturing of the fungus on potato-dextrose agar slopes. Media composition and culture conditions were the same as described earlier[8,9].

The total lipid of the mycelium was extracted with chloroform: methanol (2 : 1 v/v) and was freed from water soluble impurities by the method of Folch *et al.*[10].

Extracellular ammonia was estimated by the method of Fawcett and Scott[11].

Cell free extracts for enzyme assay were prepared by grinding the mycelial mat with half its weight of alumina in 0.1 *M* potassium phosphate buffer (*p*H 7.2) to give a 20% extract, The homogenate was centrifuged at 15000 *g* for 20 min and the supernatant was used for enzyme assays.

Protein content was determined by the method of Lowry *et al.*[12] using bovine serum albumin as standard. Avidin (Nutritional Biochemicals, Ohio) was dissolved in 0.05 *M* acetate buffer PH5 asceptically.

(NADP) Glutamate dehydrogenase — (EC 1.4.1.3) was measured by following the method of Thomulka and Moat[13]. Reaction mixture consisted or 4 μmole of ammonium sulphate. 1.7 μmole of reduced NAD or NADP, 100 μmole of tris-HCl buffer (*p*H 7.3), 20 μmole of α-ketoglutarate (*p*H 7) and enzyme preparation in a total volume of 3 ml. Ammonium sulphate was omitted in the controls and decrease in absorbance at 340 nm was recorded.

Assay of Proteolytic Activity — The proteolytic activity was assayed routinely by the method of Anson[14]. The reaction system contained 1.5 ml Hammerstein casein solution (5 mg/ml) dissolved in 0.1 M tris-HCl buffer *p*H 7.5 and an appropriate quantity of enzyme (200 μg protein) in a total system of 2 ml.

The system was incubated routinely at 50°C for one hour and the reaction terminated by 3 ml of 5 per cent TCA. After centrifuging to 1 ml of supernatant, (5 ml) of 0.4 NaOH was added followed by 1 ml of Folin's reagent. One unit of enzyme was defined as the amount of enzyme which caused change of 0.10 OD at 500 nm/660 nm per minute under the stated experimental conditions.

Results and Discussion

Earlier studies[8] in our laboratory indicated that addition of avidin to cultures of *Aspergillus nidulans* resulted in the decrease of fatty acid with resultant increase in cellular synthesis when the fungus was grown on ammonium nitrate as sole nitrogen source.

On supplementing the medium with heated egg-white, a 2-3 fold increase in the total cellular mass

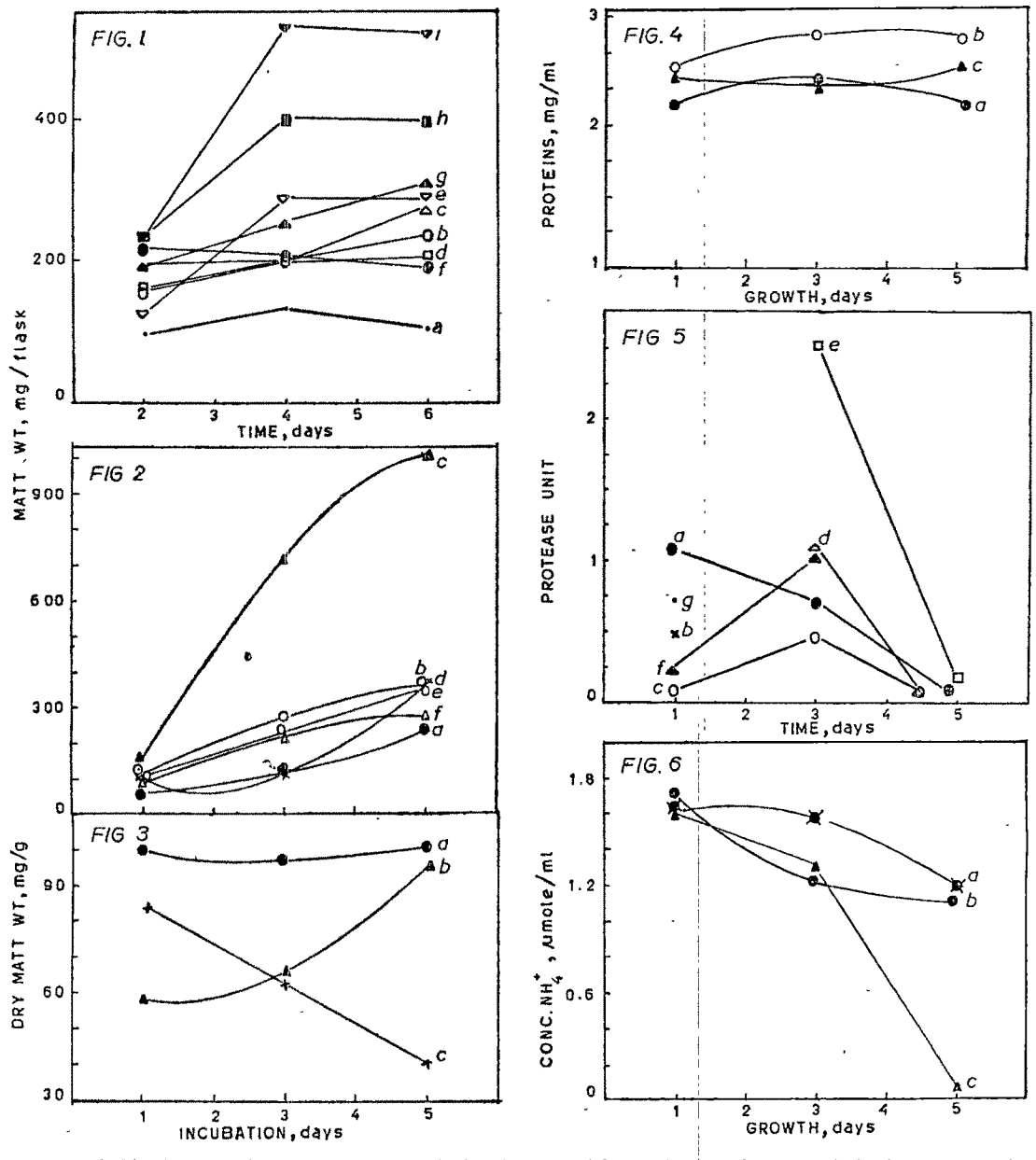

Fig. 1 — Growth kinetics on different days of incubation in *A. nidulans* using varying conc. (ml) of raw egg white [a, control; b, 0.1; c, 0.3; d, 0.5; e, 1.0 and heated inactivated egg white; f, 0.1; g, 0.3; h, 0.5; i, 1.0] Fig. 2 — Growth kinetics on different days of incubation in *A. nidulans* under different culture conditions [a, normal; b, addition of raw egg white; c, heat inactivated egg white; d, Avidin; e, heat inactivated avidin; f, heat inactivated bovine serum albumen] Fig. 3 — Total lipid content of *A. nidulans* under different culture conditions [a, normal, b, heat inactivated egg white; c, avidin] Fig. 4 — Extracellular protein profile in *A. nidulans* on different days of incubation under different culture conditions [a, normal; b, addition of raw egg white; c, heat inactivated egg white] Fig. 5 — Extracellular protein profile in *A. nidulans* on different days of incubation under different culture conditions [a, normal at *p*H 5; b, *p*H 7 2; c, addition of raw egg white at *p*H 5 and d, *p*H 7.2; e, under heat inactivated egg white added conditions at *p*H 5; f, *p*H 2.7; g, *p*H 9.0] Fig. 6 — Extracellular ammonia in *A. nidulans* on different days of incubation under different culture condition [a, normal; b, addition of raw egg white; c, heat inactivated egg white]

occurred over the control cultures, and over those to which untreated egg-white was added (Fig. 1). When egg-white was replaced with pure avidin (Nutritional Biochemicals Ohio), 5 units, there was no net increase in the total cellular mass until the fourth day of incubation but the replacement with heat-inactivated avidin, showed marked increase in the total cellular mass of the culture (Fig. 2). Although heated BSA also could result in a higher cell mass, the maximum increase of 3-fold was that by heated egg-white

TABLE 1 — GROWTH KINETICS OF *A. nidulans* UNDER DIFFERENT CULTURE CONDITIONS

[Protein content of the medium was kept at 17.818 mg/ml in terms of bovine serum albumin as standard protein]

Cultural conditions	Days of incubation — Dry mycelial Wt (mg/flask) 1st day	4th day	6th day
Normal	85	102	120
Normal + Raw egg white	127	275	239
Normal + Bovine serum albumin	143	178	215
Normal + egg white heat inactivated	236	529	520

TABLE 2 — NADP* - GLUTAMATE DEHYDROGENASE ACTIVITY IN NORMAL AND EGG WHITE SUPPLEMENTED CULTURES OF *A. nidulans*

Culture conditions	NADP-GDH Activity units/mg/protein — Days of incubation 2nd day	4th day
Normal	3.33	11.71
Normal + Raw egg white	6.09	17.75
Normal + egg white heat inactivated	16.18	38.48

*The enzyme was extracted from 2nd 4th day old cultures. Units of the enzyme is the amount which brings about a decrease of 0.001 in optical density in one minute.

(Table 1). Next it was checked whether the heat treatment of avidin or egg-white does in fact inactivate the capacity of avidin to inhibit fatty acid synthesis. 5 units of avidin, untreated and heat treated were added to different flasks and the fatty acid content of the cultures was determined.

Figure 3 shows that total lipid content of the culture decreased when untreated avidin was added, which is in agreement with the earlier observations by Mattoo *et al.*[15].

The lipid content of the cultures supplemented with heated egg white increased with increase in total cell-mass. It has been shown[16] that alteration in the native physical structure of a protein like albumin by hydrolysis, or by dilution with water results in an enhancement in the growth of test organisms. Thick albumin is a better source for growth of bacteria than thin albumin. However thin albumin supplemented with ovomucin it equally good substrate[17].

The protein composition of the thick egg-white studied by Yasushi Sato and Shigeru Hayakawa[17] consisted of gel fraction containing 0.14% insoluble ovomucin, 0.20 per cent of soluble ovomucin and 1.6% lysozyme. The concentration of egg white in terms of bovine serum albumin as reference protein was 17.818 mgs of BSA = 1 ml of egg-white/flask and showed that on supplementation of equal concentrations, bovine serum albumin could also act as an ideal substitute protein in place of egg-white.

It was surmised that feeding of a protein viz. avidin, egg-white, BSA to *A. nidulans* may result in the induction of proteases which in turn would break down the protein polymers to provide simple monomers for utilization, by the fungus to yield higher cell mass. Therefore, acid, neutral and alkaline protease levels were studied during growth on simple and complex media.

There was an induction in the proteolytic activity, though the extracellular protein concentration did not vary significantly throughout the incubation period (Fig. 4). *p*H 7.2 active protease was not active in control and heat treated egg-white added cultures but showed maximum activity by the third day of incubation in culture fed with raw egg-white. The *p*H 5 active protease showed a maximum activity on the first day of incubation and thereafter showed a steady decline in the control as the time of incubation progressed (Fig. 5).

Earlier it has been reported that there was an enhancement in the intracellular ammonium uptake and that one of the factors for higher assimilation could have been NADP-glutamate dehydrogenase.[18] Much information in literature confirms the role of glutamate dehydrogenase in ammonia assimilation by microorganisms[19-23]. Our data shows a marked decrease in extracellular ammonia from culture filtrates of heat inactivated egg-white treated cultures as compared to the control (Fig. 6). Corresponding to this change there was an increase in NADP-glutamate dehydrogenase activity in heat-treated egg-white added cultures. This may be one of the factors responsible for higher ammonia assimilation (Table 2) and this can also explain the earlier results[18].

The results presented in the present investigation suggest that the increase in cellular synthesis on addition of either untreated or heat treated egg-white to the cultures is probably due to the breakdown of the protein by proteases and that break-down products could be used as a ready made source of nitrogen. This can then bring about an enhancement in the cellular mass of the culture. The high proteolytic activity during the initial stages of incubation would make the medium of egg-white added cultures highly nutritious for the mold and then the mold at the same time can utilize more ammonia. These nitrogenous sources could then be converted into cellular protein via the ammonia assimilating route, i.e. NADP-glutamate dehydrogenase. It is thus clear that the enhancement in the total cellular mass is due to the rich source of protein present in the egg-white and permeability change brought about by the changed fatty acid pattern in the membranes of *A. nidulans* in the presence of avidin.

Results with pure avidin also showed that on inactivation the avidin brings about an increase in cell mass rather than in its active form when it can bind biotin.

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