Glutamic Acid Dehydrogenases in Quiescent and Germinating Conidia of *Neurospora crassa*

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SUMMARY

As conidia of *Neurospora crassa* aged, the activity of NADP-specific glutamic acid dehydrogenase (NADP-GDH) decreased to negligible values. Subsequent to this decrease, a significant increase occurred in the activity of the NAD-specific glutamic acid dehydrogenase (NAD-GDH). Incubation of aged conidia in basal medium resulted in over a 100-fold increase in NADP-GDH activity within 3 hr. Although no net protein synthesis occurred during these early stages of germination, a turnover in protein was observed. The data presented are consistent with de novo synthesis of NADP-GDH. Development of NADP-GDH activity was dependent upon an appropriate carbon source and pH value. An exogenous nitrogen source was not required. The data do not directly support reciprocal regulation of the synthesis of NAD-GDH and NADP-GDH in Neurospora as postulated by Sanwal & Lata (1962a, b).

INTRODUCTION

Changes in enzyme activity, presumably reflecting enzyme synthesis, in Neurospora, have been the subject of numerous investigations (Horowitz, Fling, MacLeod & Watanabe, 1961; Horowitz, 1965; Turian, 1961; 1963; Turian, Seydoux & Volkman, 1962). Changes in the activity of NADP-GDH and NAD-GDH in Neurospora are of particular interest since the published evidence indicates that these enzymes are regulated reciprocally in their synthesis. To account for this reciprocality, Sanwal & Lata (1962b) proposed that the two enzymes are under the control of a single repressor substance which is active in repressing NAD-GDH, but is active against NADP-GDH only when combined with urea which serves as a co-repressor. The 'urea effect' is postulated as being specific for stages in the development of Neurospora following germination, since Sanwal & Lata (1962a) were not able to detect NAD-GDH in conidia until germination began, at which time NADP-GDH also was increasing. This observation suggests that the regulation of the synthesis of the two GDH enzymes in Neurospora is qualitatively different during the germination process.

The data presented in this paper concerns the changes in the activity of NADP-GDH and NAD-GDH in Neurospora which occur in quiescent and germinating conidia. The changes in activities in germinating conidia were measured over a period up to 7 hr and showed that an increase in NADP-GDH enzyme was not precisely accompanied by a decrease in the activity of NAD-GDH which was found to be present in

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both quiescent and germinating conidia. These changes probably reflect de novo synthesis, rather than activation of NADP-GDH, which is independent of exogenous nitrogen but dependent on an energy source.

METHODS

Organism. The wild-type strain of Neurospora crassa used in these experiments was STA4 (an asexual derivative of St Lawrence 74A) obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire, U.S.A.

Chemicals. 2-Oxoglutarate (free acid or 0-1 m solution in phosphate buffer, pH 7-5), cis-oxalacetate (grade I), dihydricotinamide adenine dinucleotide phosphate (NADPH₂, type II), dihydricotinamide adenine dinucleotide (NADH₂, grade III) were obtained from Sigma Chemical Company, St Louis, Missouri. Amino acids and cycloheximide were obtained from the California Corporation for Biochemical Research (Los Angeles, California, U.S.A.). Uniformly labelled l-valine-¹⁴C (208.5 mc/mole) was supplied by the New England Nuclear Corporation (Boston, Massachusetts, U.S.A.). Enzyme-hydrolysed casein (N-Z-Case) was obtained from Sheffield Chemical (a division of National Dairy Products Corporation, Norwich, New York). Yeast extract and Casamino acids were obtained from Difco Laboratories (Detroit, Michigan) and Tween 80 was purchased from Atlas Powder Company (Wilmington, Delaware).

Culture media. The basal medium used in these investigations was medium N+2 % sucrose (Vogel, 1956) supplemented with trace elements (Beadle & Tatum, 1945). Deletions, additions and other modifications made to the basal medium are described in connexion with specific experiments. For certain experiments, buffers or buffers with additions were utilized (Gomori, 1955). Wainwright's medium (Wainwright, 1959) supplemented with trace elements was used for the production of conidia. For the determination of conidial viability, the basal medium was modified by the substitution of sorbose 0-1 % and glycerol 0-5 % for sucrose and the addition of peptone 0-5 %, yeast extract 0-5 % and agar 1-5 % (w/v).

Determination of conidial viability and germination. Conidial viability was determined in one of two ways. In the experiment with ageing conidia viability was ascertained by a direct count of the fraction of conidia having germ tubes after a dilute culture had been incubated for a suitable period. Evidence for germination in experiments involving the incubation of dense conidial suspensions was obtained by direct inspection of conidia for the presence of germ tubes, at the end of the experiment. However, under the conditions used throughout these studies (conidial concentrations of 1-0 to 6-2 x 10⁶ viable conidia/ml) no germ tubes were found to occur within the experimental period. Thus, in these investigations conidial viability was not equivalent to visible germination during the experiment. Under these circumstances viability was determined by plating a suitable dilution of the dense conidial suspension on supplemented sorbose + glycerol agar and counting the resulting colonies.

Preparation of large batches of conidia. The methods for obtaining large batches of conidia described by Barratt (1963) were used, with the following modifications. The inverted Fernbach flasks were kept at 32° for 36-48 hr rather than 24 hr. Subsequently the flasks were incubated at 22° and high relative humidity (range 70-85 %, mean 80 %). Aged conidia were harvested in 0-85 % NaCl solution containing 0-1 %
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Conidia were loosened from the mycelial mat with the aid of a sterile stirring bar and a magnetic stirrer, and were separated from mycelial fragments as described by Barratt (1963).

Conditions for conidial incubation. Following concentration by centrifugation at 10,500 g for 25 min. at 0°C, the total conidial wet weight was ascertained, and samples were removed for the determination of dry weight and initial enzyme activities. The remaining conidia were resuspended in a minimum volume of saline (final volume of the suspended conidia from 8 Fernbach flasks varied from 30 to 40 ml). Samples of suspended conidia were inoculated into 500 ml. Erlenmeyer flasks containing 250 ml. of the medium being tested. In two experiments with uniformly labelled l-valine-14C, 0·5 μc/ml. or 0·25 μc-ml. of the labelled valine were added to either 15 ml. or 30 ml. of medium, and in one instance to 120 ml. of medium. The inoculated flasks were aerated on a rotary shaker at approximately 150 rev./min. at 32°C for 0·5-7 hr. Following shaking, the conidia were centrifuged and a sample was removed to determine dry weight. The remainder were resuspended in 9 volumes of cold (0°C) 0·1 m-Na phosphate buffer (pH 7.0) in preparation for extraction. The extraction buffer contained 2-mercaptoethanol (1·0 mm) to stabilize the NAD-GDH activity. In those experiments involving l-valine-14C, the conidia were thrice washed in 0·85 % (w/v) NaCl solution containing unlabelled valine and then suspended in cold (0°C) buffer.

Preparation of extracts. Conidia were disrupted at maximum output for 10 min. with a model S-125 Sonifier (Branson Instruments Inc., Stanford, Connecticut, U.S.A.), generating sonic oscillations of 20 kc./sec. The instrument was equipped with a micro-tip which has a power output 3-5 times that of the standard horn. 10-15 ml. volumes were treated ultrasonically in a Rosette cooling cell (Model 25) suspended in an ice bath (−4°C to −8°C). The temperature of the extracts at the termination of ultrasonic treatment varied between 6°C and 12°C. The resulting extract was clarified by centrifugation at 17,000 g for 25 min. at 0°C (Servall Superspeed RC-2) and the supernatant fluid was assayed immediately.

Enzyme assays. All assays were done by following the oxidation of the appropriate coenzyme (NADH2 or NADPH2) in a quartz spectrophotometer cell in a Cary model 15 spectrophotometer at 340 mμ at 25°C.

NADP-GDH (l-glutamate: NADP oxidoreductase E.C. 1.4.1.4) was assayed as described previously (Barratt, 1963) with the exception that the concentration of 2-oxoglutarate was decreased by 40%. Since it has recently been shown that NADP-GDH is in the inactive form when extracted at pH 7.0 (West, Tuveson & Barratt, 1966), the supernatant fluids were pre-incubated in the presence of tris-HCl buffer (pH 7.8) and 2-oxoglutarate for 20 min., a condition shown to achieve full activation. The reaction was initiated by adding NH4Cl and NADPH2. When the free acid was used it was neutralized to pH 7-0.

The assay mixture for NAD-GDH (l-glutamate: NAD oxidoreductase E.C. 1.4.1.2) consisted of the following in a 4 ml. cell: 2·1 ml. of 0·1 m-Na phosphate buffer (pH 8·0) containing 1·0 mm-2-mercaptoethanol; 0·2 ml. of 0·1 m-2-oxoglutarate solution; 0·15 ml. of 3·0 m-NH4Cl in the phosphate buffer; sufficient volume of a 1·5 mm solution of NADH2 to contribute an extinction of 0·5 to the final reaction mixture (volume varied between 0·16 and 0·20 ml.). The reaction was initiated by adding 0·1 ml. of enzyme solution.

The assay for malate dehydrogenase (MDH; l-malate: NAD oxidoreductase
E.C. 1.1.1.37) was a modification of the procedure described by Munkres (1965). The assay mixture consisted of the following in a 4 ml. cell: 2.25 ml. of 0.111 M-K phosphate buffer (pH 7.4); 0.2 ml. of 0.012 M-cis-oxalacetate solution; sufficient volume of a 1.5 mM solution of NADH₄ to contribute an extinction of 0.5 to the final reaction mixture (volume varied between 0.16 and 0.20 ml.). The reaction was initiated by adding 0.1 ml. of enzyme solution.

Since crude preparations were assayed in all but one experiment, correction for non-specific oxidation of the coenzymes was made by subtracting rates obtained in the absence of substrate.

One enzyme unit is defined as that amount of enzyme which will catalyse the transformation of 1 μmole substrate/min. A unit of enzyme activity is equivalent to a change in extinction at 340 μm of 2.27/min. For convenience, the activities are presented in terms of milli-units (m.u.).

Protein determinations. Protein determinations were made on the soluble proteins by the Folin phenol method of Lowry, Rosebrough, Farr & Randall (1951). Specific activities are expressed as m.u./mg. protein.

Counting of radioactive samples. Supernatant material from ultrasonically treated conidia incubated in the presence of L-valine-¹⁴C was spotted on 2.3 cm. filter-paper disks and treated by a modification of the procedure described by Mans & Novelli (1961). Disintegrations were detected in a liquid scintillation counter (Nuclear Chicago model 725, Nuclear-Chicago Corporation, Des Plaines, Illinois).

Electrophoresis. The methods used for electrophoresis on cellulose acetate strips were as described previously (Barratt & Strickland, 1963).

RESULTS

Effects of conidial ageing.

Preliminary experiments had suggested that NAD-GDH and NADP-GDH activity in conidia varied with conidial age (West et al., 1966; Barratt et al., 1966; Stine, 1966). A systematic investigation was undertaken to determine NAD-GDH and NADP-GDH in conidia from cultures of different ages. Malate dehydrogenase (MDH) was also assayed in the extracts obtained from the conidia to determine whether an enzyme metabolically separated from the glutamate dehydrogenase system varied with conidial age. The results of this experiment are presented in Table 1. Four days after inoculation was the earliest time when sufficient conidiation had occurred to permit harvesting. NADP-GDH activity decreased dramatically in conidia between 4 and 8 days of age. The inoculum for the 4-, 12-, 14-, 18- and 21-day cultures was prepared from a single agar slope. The inoculum for the remaining cultures was prepared from a duplicate slope 3 days previously. The slightly elevated NADP-GDH activities in the 14- and 18-day cultures may indicate some slight difference in the duplicate agar slopes used for inoculum which affected the decline of NADP-GDH during ageing. However, in more than 20 experiments NADP-GDH activity in 21-day (or older) conidia was never observed to be significantly greater than that reported for the 21-day culture in Table 1 (less than 2.5% of that in young conidia). The NADP-GDH activity was determined in 4-day conidia in four independent experiments (see footnote, Table 1). It is apparent from Table 1 that the extracts of Neurospora conidia did exhibit significant NAD-GDH activity, irrespective of culture age.
and contrary to the observations of Sanwal & Lata (1962a). The NAD-GDH activity remained relatively constant in conidia up to 14 days, after which there was a marked increase.

Table 1. The relationship of culture age to specific activity in crude extracts of conidia of Neurospora crassa sta 4

<table>
<thead>
<tr>
<th>Culture age (days)</th>
<th>Protein (mg./g. dry wt.)</th>
<th>Specific activity (m.u./mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NADP-GDH</td>
</tr>
<tr>
<td>4*</td>
<td>158</td>
<td>278</td>
</tr>
<tr>
<td>8</td>
<td>173</td>
<td>15:1</td>
</tr>
<tr>
<td>12</td>
<td>173</td>
<td>17:7</td>
</tr>
<tr>
<td>13</td>
<td>200</td>
<td>2:7</td>
</tr>
<tr>
<td>14</td>
<td>164</td>
<td>11:2</td>
</tr>
<tr>
<td>15</td>
<td>219</td>
<td>2:7</td>
</tr>
<tr>
<td>16</td>
<td>179</td>
<td>2:7</td>
</tr>
<tr>
<td>17</td>
<td>174</td>
<td>1:8</td>
</tr>
<tr>
<td>18</td>
<td>147</td>
<td>10:6</td>
</tr>
<tr>
<td>20</td>
<td>166</td>
<td>4:4</td>
</tr>
<tr>
<td>21†</td>
<td>114</td>
<td>6:2</td>
</tr>
<tr>
<td>23†</td>
<td>54</td>
<td>6:2</td>
</tr>
</tbody>
</table>

* Specific activities for NADP-GDH in crude extracts of conidia from 4-day cultures determined in 3 additional experiments were: 228, 372, 382.
† Extractable protein from 21- to 23-day conidial cultures averaged 153 mg./g. dry weight (Tables 2–6).

The MDH activity in the conidial extracts was unrelated to conidial age.
Parallel to these enzyme activity studies in ageing conidia, conidial viability was determined. Conidia from the 4-day culture showed essentially 100% germination within 6 hr, in agreement with the findings of Ryan (1948). The % germination remained high (85%) for cultures up to 18 days old. However, as the cultures aged, there was a marked tendency for germination to be non-uniform and delayed. Thus the conidia from the 23-day culture showed visible germination only after incubation for 8 hr. In such conidia germination increased up to 12 hr, by which time mycelial masses began to interfere with counting. For conidia from the 23-day culture, it was estimated that germination was between 50 and 70%. Thus, ageing resulted in some loss of conidial viability.

Observations on incubating aged conidia

Neurospora mycelium shows appreciable degrees of NADP-GDH activity (Barratt, 1963). This activity developed during the process of germination or shortly thereafter. Therefore, aged conidia were incubated in basal medium for up to 3 hr and the NADP-GDH activity assayed. After 3 hr of incubation no visible evidence of germination was observed at the conidial concentrations used. Neither the initial inoculum nor conidia incubated for 0.5 hr in basal medium exhibited significant NADP-GDH activity (Fig. 1; Table 2). After shaking for 3 hr, however, NADP-GDH activity increased over 100-fold (Table 2). It was also apparent that absence of the carbon source (sucrose) severely restricted the development of NADP-GDH activity. The activity which did develop was probably supported by carbon reserves in the conidia. The omission of a nitrogen source from the basal medium did not decrease the
development of NADP-GDH activity significantly. The simultaneous omission of both carbon and nitrogen sources gave an NADP-GDH activity equivalent to that found when a carbon source was omitted. In this experiment the extractable soluble protein decreased after the first 0.5 hr of shaking and remained low for all treatments up to 3 hr. This initial decrease in extractable protein was observed in all experiments. Although the absolute quantity of extractable protein varied from experiment to experiment, the decrease in treated conidia shaken for as long as 4 hr, as opposed to untreated conidia, remained qualitatively equivalent. The NAD-GDH activity did not vary significantly throughout the experiments (Tables 2-6).

The kinetics of NADP- and NAD-GDH development were followed over a 4 hr period in basal medium. As expected, in the first 0.5 hr there was little development of NADP-GDH activity, but there was a marked decrease in extractable protein (Fig. 1). From 1-0 hr on, the development of NADP-GDH activity appeared to be linear with time. These same kinetics were observed in two similar experiments. Although the amounts of extractable protein varied widely in this experiment, they were consistently lower for the treated conidia up to 4 hr than for the conidia of the inoculum. The fact that the specific activity of NADP-GDH increased in a regular manner suggests that the variations in extractable protein were random, except for the initial decline, a fact also confirmed in subsequent experiments (Fig. 1; Tables 2-6). During the 4 hr period of incubation there appeared to be a slight decline in NAD-GDH activity, beginning between 1-5 and 2-0 hr.
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Subsequent experiments were directed towards determining what other factors might influence the development of NADP-GDH in incubating aged conidia. Substitution of phosphate buffer (pH 5-7) + sucrose for basal medium resulted in an NADP-GDH activity approximately 60% of that attained in the basal medium in 3 hr (Table 3). The addition of trace elements to the pH 5-7 buffer + sucrose increased NADP-GDH activity, but still not to the degree attained in the basal medium. Similar experiments with phosphate buffer (pH 7-0) + sucrose resulted in negligible NADP-GDH activity, while in citrate + phosphate buffer (pH 5-0) + sucrose NADP-GDH activity was

Table 2. NADP-GDH specific activity in crude extracts of conidia of Neurospora crassa sta 4 shaken in various media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time of shaking (hr)</th>
<th>Protein (mg./g. dry wt)</th>
<th>Specific activity (m.u./mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
<td>0</td>
<td>160</td>
<td>1.7</td>
</tr>
<tr>
<td>Basal medium</td>
<td>0-5</td>
<td>58</td>
<td>4.4</td>
</tr>
<tr>
<td>Basal medium minus sucrose</td>
<td>3-0</td>
<td>67</td>
<td>300</td>
</tr>
<tr>
<td>Basal medium minus NH₄NO₃</td>
<td>3-0</td>
<td>95</td>
<td>280</td>
</tr>
<tr>
<td>Basal medium minus sucrose</td>
<td>3-0</td>
<td>87</td>
<td>32</td>
</tr>
<tr>
<td>Basal medium minus NH₄NO₃</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Vogel's minimal N containing 2% sucrose (58.5 m).
about 40% of that attained in the basal medium in 3 hr. Again NAD-GDH activity showed no significant changes within 3 hr, irrespective of treatment. However, when the conidia had been shaken for 7 hr there was a decided increase in extractable protein, accounting for the exhibited decline in NAD-GDH specific activity.

The quantitative requirements for sucrose in the induction of NADP-GDH activity were investigated. 5.85 and 58.5 mM-sucrose supported equally the development of NADP-GDH activity (Table 4). The minimal concentration of sucrose necessary for maximal development was found (see Table 5). This experiment, in which phosphate buffer (pH 5.7) + sucrose was used in place of basal medium, showed that the limiting sucrose concentration was between 0.58 and 1.45 mM.

Table 4. NADP-GDH and NAD-GDH specific activities in crude extracts of conidia of Neurospora crassa sta 4 shaken in variously modified basal media

Viable conidia 4.6 x 10^7/ml. from a 21-day culture were inoculated into each flask containing 250 ml. of medium.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Time of shaking (hr)</th>
<th>Protein (mg/g. dry wt.)</th>
<th>Specific activity (m.u./mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
<td>0</td>
<td>147</td>
<td>4.4 119</td>
</tr>
<tr>
<td>Sucrose (58.5 mM)</td>
<td>3.0</td>
<td>80</td>
<td>800 96</td>
</tr>
<tr>
<td>Sucrose (5.85 mM)</td>
<td>3.0</td>
<td>89</td>
<td>920 110</td>
</tr>
<tr>
<td>Sucrose (58.5 mM)</td>
<td>4.0</td>
<td>77</td>
<td>860 92</td>
</tr>
<tr>
<td>Na acetate (58.5 mM)</td>
<td>3.0</td>
<td>81</td>
<td>58 150</td>
</tr>
<tr>
<td>Na acetate (5.85 mM)</td>
<td>3.0</td>
<td>115</td>
<td>174 109</td>
</tr>
<tr>
<td>Sucrose (58.5 mM) + Casamino</td>
<td>3.0</td>
<td>69</td>
<td>380 97</td>
</tr>
<tr>
<td>acids (1.4%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose (58.5 mM) + Casamino</td>
<td>3.0</td>
<td>64</td>
<td>420 94</td>
</tr>
<tr>
<td>acids (2.8%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose (58.5 mM) + arginine</td>
<td>3.0</td>
<td>87</td>
<td>390 120</td>
</tr>
<tr>
<td>(3.2 mM) + lysine (6.8 mM) +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>threonine (5.0 mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The role of other carbon sources and certain amino acids in the induction of NADP-GDH activity was examined. When Na acetate was substituted for sucrose no appreciable development of NADP-GDH occurred. In this experiment the highest concentration of Na acetate (58.5 mM) exhibited NADP-GDH activity approximately 33% of that found with the low concentration of Na acetate (5.85 mM), neither activity being comparable to that attained in buffer + sucrose or in the basal medium (Table 4). This experiment was repeated with phosphate buffer to which Na acetate was added at three concentrations. The results confirmed the original observations and indicated that the inhibition by Na acetate was not peculiar to the addition of Na acetate to basal medium in place of sucrose (Table 6). MDH was also assayed in this experiment to verify the result (Table 1) that its activity did not vary systematically under conditions which allowed the development of NADP-GDH activity. The results of this experiment were unusual in that after 3 hr of shaking conidia in the basal medium and in buffer + sucrose, the NADP-GDH activities found were identical within the limits of experimental error. However, if one takes the NADP-GDH activity at 4 hr and constructs a graph using 0.8 hr as the point where NADP-GDH activity should begin to develop (Fig. 1), the 3 hr point determined experimentally
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was about 50% under what might have been predicted. It seems probable that the NADP-GDH specific activity determined for conidia shaken for 3 hr in basal medium was low as the result of a technical error. If this were true, then the experiment would

Table 5. NADP-GDH and NAD-GDH specific activities in crude extracts of conidia of Neurospora crassa sta 4 shaken in various media

Viable conidia 10⁷/ml from a 21-day culture were inoculated into each flask containing 250 ml of medium. All cultures were shaken for 3 hr with the exception of the untreated conidia which were not shaken.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Medium</th>
<th>Protein (mg/g. dry wt)</th>
<th>NADP-GDH</th>
<th>NAD-GDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7)</td>
<td>143</td>
<td>88</td>
<td>20</td>
<td>170</td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7)+ sucrase (5-85 mm)</td>
<td>68</td>
<td>200</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7)+ sucrase (2-9 mm)</td>
<td>74</td>
<td>240</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7)+ sucrase (1-45 mm)</td>
<td>100</td>
<td>254</td>
<td>183</td>
<td></td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7)+ sucrase (0-58 mm)</td>
<td>63</td>
<td>130</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7)+ sucrase (5-85 mm)+ biotin (0-08 pg./ml.)</td>
<td>65</td>
<td>240</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7)+ glucose (5-8 mm)</td>
<td>68</td>
<td>170</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

Table 6. NADP-GDH, NAD-GDH and MDH specific activities in crude extracts of conidia of Neurospora crassa sta 4 shaken in various media

Viable conidia 2.7 x 10⁷/ml. from a 21-day culture were inoculated into each flask containing 250 ml of medium.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time of shaking (hr)</th>
<th>Protein (mg/g. dry wt)</th>
<th>Specific activity (m.u./mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum</td>
<td></td>
<td></td>
<td>NADP-GDH</td>
</tr>
<tr>
<td>Basal medium* (5-85 mm sucrose)</td>
<td>0</td>
<td>181</td>
<td>0.9</td>
</tr>
<tr>
<td>Basal medium</td>
<td>3-0</td>
<td>118</td>
<td>176</td>
</tr>
<tr>
<td>Basal medium</td>
<td>4-0</td>
<td>Not determined</td>
<td>430</td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7)</td>
<td>1-5</td>
<td>100</td>
<td>1.9</td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7) + Na acetate (58-5 mm)</td>
<td>3-0</td>
<td>42</td>
<td>25</td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7) + Na acetate (5-85 mm)</td>
<td>3-0</td>
<td>68</td>
<td>160</td>
</tr>
<tr>
<td>0.1 M-phosphate buffer (pH 5.7) + Na acetate (1-0 mm)</td>
<td>3-0</td>
<td>59</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Vogel's minimal N containing 2% sucrose (58-5 mm) except where indicated.

conform to all other experiments in which buffer + sucrose was always less effective in supporting the development of NADP-GDH than was the basal medium (Table 3 and unpublished experiments). In another experiment glucose was used in place of
sucrose, and buffer + sucrose was further supplemented with biotin (0·08 μg/ml.) in another case. If sucrose, pH value and biotin were the critical factors in the basal medium allowing for the development of maximal NADP-GDH activity, the addition of biotin would be expected to double the NADP-GDH activity. However, biotin did not enhance the effect of sucrose alone and equimolar glucose did not support the development of NADP-GDH activity as well as did sucrose. Concurrent with the experiment in which Na acetate was substituted for sucrose in basal medium, certain amino acids were tested for their effects on NADP-GDH activity. As can be seen from Table 4, Casamino acids or a mixture of amino acids in the basal medium depressed the development of NADP-GDH about 50%. The NAD-GDH activity again remained about the same in most of the extracts, showing what can only be interpreted as random fluctuations.

Two experiments were done in an attempt to explain the differences in degrees of NADP-GDH activity attained in basal medium in different experiments after 3 hr shaking (Tables 2-4). In the first of these, the speed of the shaker was increased to 180 rev./min. from the usual 150 for one set of flasks, and decreased to 114 rev./min. for a second set. Shaking for 1·5 or 3·0 hr at these two speeds showed that the NADP-GDH activities attained were not significantly different. The amounts of viable conidia varied from experiment to experiment, as indicated in the individual tables; this might explain the observed differences in final NADP-GDH activity. The conidial concentration was varied systematically in the basal medium. The results indicated that NADP-GDH activity was restricted in its development when the conidial concentration exceeded \(6.2 \times 10^7\) viable conidia/ml. Since concentrations above this were not used in any experiments detailed in this paper, the variations in maximal NADP-GDH activity cannot be attributed to variations in viable conidial concentration.

In summary, sucrose appeared to be the most effective carbon source, and some other factor, as yet undetermined, in the basal medium other than sucrose in concert with the proper pH value is essential for maximum NADP-GDH development. Shaking speed and the concentration of viable conidia were not important factors in the experiments reported here.

Evidence for de novo NADP-GDH synthesis in incubating conidia

The effect of inhibition of protein synthesis on the observed changes in NADP-GDH activity was investigated. The addition at 1·5 hr (Fig. 1) or 2 hr (Table 3) of cycloheximide (10 μg/ml.), which effectively inhibits protein synthesis in fungi (Haidle & Storeck, 1966; Pall, 1966), resulted in the total cessation of development of NADP-GDH activity. Sodium azide (5 μM), which inhibits oxidative phosphorylation and thus secondarily protein synthesis, markedly restricted the further development of NADP-GDH activity when added at 2 hr. Such data, while suggestive, do not constitute unequivocal proof that NADP-GDH was being synthesized de novo during the incubation period. It was essential to show that protein synthesis occurred during the standard incubation period. L-valine-\(^{14}\)C incorporation was selected to ascertain protein turnover during incubation. Before doing an experiment with L-valine-\(^{14}\)C, it was necessary to investigate the effects of L-valine on the development of NADP-GDH activity. In two experiments it was found that L-valine used at concentrations equivalent to that of the labelled valine in one experiment, and twice the concentration of the label in a second experiment, restricted maximal NADP-GDH
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Development by about 50%. The inhibition was not sufficient to preclude the proposed experiment. Since the amino acid inhibition of NADP-GDH development seemed rather non-specific (Table 4), no attempt was made to find an amino acid other than valine to use in the proposed labelling experiment.

Conidia from a 21-day culture were incubated in basal medium containing L-valine-14C and either kept cold in an ice bath with constant stirring or shaken for 4 hr with or without cycloheximide (10 µg./ml.) at 32°C (Table 7). The conidia in basal medium exhibited a significant uptake of L-valine-14C, substantial (85.8%) incorporation of the label into protein (measured as trichloroacetic acid-precipitable material), and a large increase in NADP-GDH specific activity. The culture containing cycloheximide showed considerable uptake of 14C, but negligible incorporation into protein, and a very low specific activity of NADP-GDH. The control flask maintained at 0°C showed negligible uptake of 14C, less than 1% incorporation into protein, and again a negligible NADP-GDH specific activity.

Table 7. Evidence for protein synthesis in incubating conidia from a 21-day culture of Neurospora crassa STA 4

Viable conidia 5.1–6.2×107/ml. from a 21-day culture were inoculated into each flask. Experimental conditions: 4 hr incubation in basal medium + 0.15 mg./ml. L-valine-14C (U.L.). Each flask contained 0.25 mc. L-valine-14C/ml.

<table>
<thead>
<tr>
<th>Incubation temp.</th>
<th>Medium</th>
<th>Total c.p.m.*</th>
<th>TCA insoluble c.p.m. †</th>
<th>Incorporation (%)</th>
<th>Specific activity (m.u./mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Basal†</td>
<td>296-0</td>
<td>2-6</td>
<td>0-90</td>
<td>Negligible</td>
</tr>
<tr>
<td>32</td>
<td>Basal ‡</td>
<td>5077-9</td>
<td>52-2</td>
<td>1-03</td>
<td>Negligible</td>
</tr>
<tr>
<td>32</td>
<td>Basal</td>
<td>9524-1</td>
<td>8170-8</td>
<td>85-80</td>
<td>190</td>
</tr>
</tbody>
</table>

* Per 100 µl. supernatant fluid of sonically treated conidia.
† TCA insoluble fraction from 100 µl. of sonically treated conidia (by a modification of the method of Mans & Novelli, 1961).
‡ Vogel's minimal N containing 2% sucrose (58.5 m).

NADP-GDH extracted from the conidia incubated with L-valine-14C at 32°C for 4 hr was purified tenfold and subjected to electrophoresis on cellulose acetate strips under conditions known to permit migration of NADP-GDH. The cellulose acetate strip was sectioned, each section divided in half, one half counted on a scintillation counter, and the other half assayed for enzyme activity. Enzyme activity was found to be associated with a diffuse radioactive peak. This result does not preclude the possibility that association of radioactivity and NADP-GDH activity might represent contamination of the unlabelled NADP-GDH by another radioactive protein. However, the isotope and protein synthesis inhibitor experiments strongly suggest that NADP-GDH was synthesized de novo during the incubation period, and thus was induced, in the sense defined by Jacob & Monod (1961).
DISCUSSION

*Neurospora crassa* conidia retain a viability of greater than 50% when stored for 23 days under conditions of high humidity and constant temperature. High humidity has been shown to maintain conidial viability in other investigations (Klingmüller 1963). Ageing conidia rapidly lost NADP-GDH activity, decreasing to a minimal value within 15 days. At that time NAD-GDH activity began to increase. Malate dehydrogenase (MDH) activity remained constant during the course of conidial ageing. Extractable protein exhibited only random fluctuations during conidial ageing; the two values obtained in the ageing experiment for 21- and 23-day conidia are considered to be spurious in the light of other experiments (see footnote to Table 1). Despite the constancy of total extractable protein during the ageing process, the changes in enzyme activity indicate that significant selective protein turnover was taking place; and that the degradation of NADP-GDH was not reciprocal with the appearance of NAD-GDH, either in magnitude or in the time course of the event.

In incubating conidia the enzyme complement is undergoing rapid change, presumably in preparation for germination. This was reflected by the marked decrease in extractable protein coincident with the rapid increase in NADP-GDH within 3 hr. The synthesis of NADP-GDH was dependent upon an exogenous carbon source (Tables 2, 3, 5, and 6). The carbon requirement was notable in that the concentration required for maximal induction was only 0.05%. This might be interpreted as meaning that the sucrose was primarily acting to produce a specific metabolite such as NADPH (through the dehydrogenation of glucose-6-phosphate) or 2-oxoglutarate, which in turn could serve as a specific inducer of NADP-GDH. Acetate, which would also be expected to serve as an energy source, was much less effective in the induction process. Acetate is known to induce the glyoxalate cycle in *Neurospora* (Turian, 1963), and this would be likely to result in diminished intracellular concentrations of 2-oxoglutarate as well as a decrease in glucose catabolism.

Exogenous ammonium ions neither repressed nor de-repressed NADP-GDH (Table 2), while exogenously supplied amino acids restricted NADP-GDH development (Table 4). The fate of the amino acids derived from the protein degraded before the induction of NADP-GDH was not investigated but the following possibilities may be considered: (a) deamination, (b) expansion of the free amino acid pool, (c) rapid conversion of amino acids into insoluble structural proteins. The only possibility which is difficult to reconcile with the data about exogenously supplied nitrogen sources is (b), the expansion of the amino acid pool. One analysis of a soluble protein fraction did not show significant quantities of free amino acids. Further investigations on the role of exogenously supplied amino acids as well as the fate and function of endogenously produced amino acids are in progress.

Although no net protein synthesis occurs within the first 4 hr of incubation, l-valine-$^{14}$C incorporation experiments established that protein turnover was taking place during this time; it is precisely during this time that NADP-GDH activity increased. The question remained whether the increase represented de novo synthesis of enzyme or activation of pre-existing enzyme. Since cycloheximide prevented the incorporation of l-valine-$^{14}$C into protein and simultaneously prevented the appearance of NADP-GDH activity (Tables 3, 7; Fig. 1), these data are consistent with de novo synthesis of NADP-GDH. However, proof of synthesis would require the
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demonstration of radioactive label associated with NADP-GDH. Electrophoresis of a partially purified preparation obtained from conidia exposed to L-valine-14C showed radioactivity associated with the region of NADP-GDH activity, but this can still not be considered unequivocal evidence since the label was distributed in overlapping bands along the cellulose acetate strip. The synthesis of NADP-GDH in the absence of net protein synthesis is not a unique situation in Neurospora. It has also been shown that the tyrosinase in Neurospora is synthesized de novo under conditions in which net protein synthesis is prevented by starvation or by certain inhibitors of growth (Horowitz et al. 1961).

In contrast to the report of Sanwal & Lata (1962a), the present experiments have shown that NAD-GDH was present in conidia under all conditions investigated. As conidia aged, in fact, the specific activity of NAD-GDH increased while the NADP-GDH activity decreased to negligible values. Since they were unable to detect NADP-GDH in conidia, Sanwal & Lata (1962b) postulated that conidia are qualitatively different with regard to the regulation of NAD-GDH and NADP-GDH. The postulated uniqueness of conidia was required since NADP-GDH and NAD-GDH were thought to be synthesized together during the process of germination, but the occurrence of these same enzymes appeared to be regulated reciprocally in mycelium. The data presented here concerning the early stages of germination clearly show that parallel synthesis of the two enzymes did not occur. It is still possible, however, that there is a difference between conidia and mycelium, in that the two enzymes do not appear to be reciprocally regulated in germinating conidia. Since it has recently been shown the NADP-GDH is an allosteric protein (West et al. 1966; West, Tuveson, Barratt & Fincham, 1967), further investigation of the inter-relationships in the regulation of NADP-GDH and NAD-GDH may be profitably investigated at the level of activity as well as synthesis.

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