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Hyperproduction of Some Glycosidases in *Neurospora crassa*

David Sternberg and A. S. Sussman

Department of Botany, University of Michigan, Ann Arbor, Michigan 48104

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Abstract. Results of a cross betwen a hyperderepressed strain (89601a) and a normal strain (74A) of *Neurospora crassa* suggest that there is a single gene difference in this trait. The evidence is clearest with amylase although a similar segregation pattern is suggested for invertase and, perhaps, trehalase. On the other hand, phosphatase activity is not affected by this gene. The gene for hyperderepression does not appear to be widespread in wild-type strains of this organism for, in the seven tested, only 89601a was hyperderepressed for amylase. The action of the hyperderepression gene probably is not due to diminished sensitivity to catabolite repression because the synthesis of amylase begins at roughly the same point in glucose depletion in both the hyperderepressed and normal strains. Furthermore, growth of these strains on constant but low levels of a carbon source causes derepression to the degree expected in both strains. Nor does hyperderepression appear to be due to achange in sensitivity to induction, according to experiments with cellobiase, which is inducible. Increases in enzyme activity due to the gene for hyperderepression are in the order, amylase-cellobiase > invertase > trehalase. Thus, the gene exhibits polarity as well as being pleiotropic. An explanation for its effect is proposed, based upon changes in the cell surface.

Key words: Neurospora crassa — Hyperderepression Gene — Amylase — Invertase.

A mutant of *Neurospora crassa* has been described by Gratzner and Sheehan (1969) which is hyperderepressed for amylase and invertase upon the depletion of the carbon source in the medium. Although the genetic data were not complete, the ability of the mutant to form large amounts of these enzymes appeared to segregate as a single gene, which has been labeled *exo-1*, and it affects the ratio of galactosamine to glucosamine found in hydrolysates of cell walls.

While studying extracellular glycosidases in this organism we discovered that the inositolles (*inos*) strain that we were using in mutant hunts also is hyperderepressed for these enzymes, as well as for cellobiase. Gratzner and Fass (1968) are quoted as having reported that this strain is derepressed for amylase and that *inos*$^+$ revertants are not derepressed. These and other properties of this strain suggested a parallel with the mutant reported by Gratzner and Sheehan. Therefore, the

response of our strain to catabolite repression was studied, along with the genetics of hyperderepression and other aspects of the functioning of this gene so that it could be compared with that described by these other workers. In addition, an hypothesis to explain its action is advanced, based upon these results and those of others.

Methods

Stock Cultures. All stocks were obtained from the Fungal Genetics Stock Center. The strain which is hyperderepressed is 89601a (*inos*), having a mixed genetic background. Strain 74A was used as a standard wild-type and served as the female parent in crosses with strain 89601a. Other wild-type strains used were Emerson (Em 5256), Chilton (a), Lein (7A), Yale (SY7A), Lindegren (1) and Abbott.

Media. Vogel's N-salts (Vogel, 1956) supplemented with 5 mg inositol/ml was used throughout as the growth medium. Except where noted otherwise, 4% glucose was added as a carbon source to maintain cultures in a repressed condition; Vogel's medium without a carbon source was used to bring cultures into the derepressed state. In order to prevent conidiation, $CaCl_2$ was omitted from the medium when enzyme levels of the progeny from the cross, 74A × 89601a, were studied. Difco corn meal agar served as the crossing medium.

Growth Conditions. All cultures except those used in the time-course studies, were grown on a rotary shaker (180 rpm) at 20—21° C in 125 ml flasks containing 30 ml medium. Derepression was initiated by decanting the medium, rinsing the cultures several times with sterile distilled water, and adding the derepressing medium. For time-course studies 10—13 cultures were grown in 125 ml flasks as above for several days, then transferred to a Fernbach flask containing 1.1 of medium with 0.75% glucose after which the pooled cultures were placed in a rotary shaker at 20° C for the duration of the time-course study.

Time-Course Experiments. In order to take closely timed samples for time-course studies of enzyme levels, a simple, automatic sampling device was constructed. A thick-walled capillary tube, with a glass wool filter at the end, extended to near the bottom of the Fernbach flask containing the culture. The capillary tube was connected to a fraction collector by 0.034 inch (i. d.) tubing. A siphon was started and the rate of flow adjusted to about 1.5 ml/hr with a Hoffman clamp. Because of the narrow diameter of the delivery system, the dead volume was kept to a minimum. The fraction collector was set to cycle every 2 hrs.

Extraction Procedures. For determination of enzyme levels, mycelia were ground with sand and cold buffer (0.05 M phosphate) in a mortar and pestle. During the experiments when randomly isolated ascospores were analyzed, the mycelia were first acetone-dried and the dry weights determined before the enzymes were extracted.

Assay Procedures. Trehalase assays were carried out in 0.05 Na-phosphate buffer at pH 5.4; 1 ml of trehalose solution (5 mg/ml) was added to 1 ml of enzyme preparation and incubated 30 min at 37° C. The reaction was stopped by adding 2 ml of the Somogyi reagent, and the glucose released was measured by a modification of the Nelson-Somogyi reducing sugar procedure (Somogyi, 1952). Invertase was measured in the same way as trehalase except that sucrose was used as the substrate and the buffer was 0.05 M acetate at pH 5.0. Glucoamylase was assayed in 0.01 M Na-phosphate buffer at pH 6.0 with 0.15% soluble starch as substrate and after 30 min at 37° C, the reaction was stopped by placing the reaction mixture

in a boiling water bath for 5 min. Glucose released from the starch was measured by the Glucostat method (Worthington Biochemical Corp., Freehold, New Jersey). Alkaline phosphatase was measured by the method of Bergmeyer (1963). The glucose level remaining in the medium during time-course studies was measured by the Nelson-Somogyi method. Protein was determined by the method of Lowry *et al.* (1951). "Cellobiase" activity was measured similarly to glucoamylase except that cellobiose (5 mg/ml) was the substrate and 0.05 M Na-phosphate at pH 5.4 was used as the buffer.

Electrophoretic Techniques. Invertase isozymes were electrophoretically separated by an Ortec pulsed power electrophoresis unit (Ortec Inc., Oak Ridge, Tenn.). An 8 to $4^1/_2$% gradient acrylamide gel was cast following the Ortec procedure (Anonymous, 1970) for the tris/citrate buffered gel system, with an ammonium persulfate catalyst. Glucose oxidase (Sigma Chemical Co., *A. niger*, type V) was incorporated into the gel at a level of 20 units/ml of the gel. The tank buffer used was the tris/borate system specified in the Ortec procedure as were the power settings during electrophoresis. After electrophoresis for about 1 hr the invertase isozymes were developed by immersing the gel at room temperature in a solution containing 0.175 g sucrose, 0.0165 nitro-blue tetrazoleum, 0.0105 g phenazine methosulfate and 2600 units of glucose oxidase in 100 ml 0.01 M NaH_2PO_4. If more rapid development was desired, incubation was carried out at 37° C. After the bands had developed to the desired intensity, the gel was rinsed several times with water and stored in 7% acetic acid.

Results

That there is a large difference in the activity of certain glycosidases in strains 89601a and 74A is shown in Table 1. The data on intracellular enzyme activity reveal that smaller differences in trehalase activity occur in the two strains than in amylase and invertase, and the former shows the largest differences among the last two enzymes. Intracellular invertase appears earlier and is more active than either amylase or trehalase. Intracellular amylase activity in strain 89601a is more than 1.000-times greater than found in strain 74A after 5 days of growth but the ratio falls to about 27:1 at 8 days. By contrast, intracellular invertase activity in strain 89601a never exceeds 13-fold that in strain 74A, and trehalase in the former strain reaches 3.5-fold more activity than in strain 74A but usually its average is closer to 2-fold or less.

Extracellular amylase activity appears earlier and in higher amounts than its intracellular equivalent but the opposite is true for trehalase and invertase. In general, the difference between strains 89601a and 74A in the production of amylase is greater in the case of the intracellular activity than the extracellular. However, the situation is reversed in the case of invertase after the sixth day of growth.

The per cent of the total activity that is extracellular is calculated from the preceding data and summarized in Table 2. It can be seen from these data that almost 90% of the amylase activity is extracellular, in contrast to trehalase whose extracellular component does not rise above about two per cent until after the sixth day of growth. Less than

Table 1. Intracellular and extracellular glycosidase activity in shaken cultures of hyperderepressed (89601a) and wild-type (74A) strains of *Neurospora crassa*

These experiments were performed with shaken cultures grown in Vogel's minimal medium with glucose for up to 8 days. Flasks were harvested daily and enzymes from the mycelium were assayed to determine intracellular activity and from the medium to yield extracellular activity

Day	Strain	Residual sugar	Protein mg/ml	Intracellular specific activity			Extracellular specific activity		
				Trehalase	Amylase	Invertase	Trehalase	Amylase	Invertase
1	89601a	1.050	4.6	3	0	1 370	0	26	33
	74A	1.025	4.0	9	0	1 450	0	30	80
2	89601a	0.028	4.7	41	30	4 043	0	140	217
	74A	0.060	4.0	18	0	2 200	0	75	158
3	89601a	0.024	4.3	186	105	9 535	7	1 128	642
	74A	0.024	4.7	426	0	5 106	3	332	663
4	89601a	0.024	4.3	349	1 977	24 422	0	11 163	2 093
	74A	0.025	3.9	385	8	6 667	0	538	1 015
5	89601a	0.024	4.0	1 800	8 750	66 250	11	43 800	3 600
	74A	0.027	4.0	550	8	7 750	0	435	800
6	89601a	0.028	3.6	2 833	11 667	93 056	54	81 667	7 333
	74A	0.025	2.6	2 289	315	13 462	6	4 108	1 270
7	89601a	0.029	2.4	4 375	16 250	133 333	250	180 000	21 000
	74A	0.028	2.4	1 729	154	10 417	0	2 450	1 250
8	89601a	0.035	2.4	5 417	20 083	191 662	1125	265 000	55 000
	74A	0.031	1.9	4 947	758	20 316	237	15 789	4 420

Table 2. Per cent of total glycosidases produced extracellularly by shaken cultures of a hyperderepressed (89601a) and wild-type (74A) strain of *Neurospora crassa*. Data calculated from Table 1

Day	Strain	% Extracellular activity		
		Trehalase	Amylase	Invertase
1	89601a	0	100	2.3
	74A	0	100	5.2
2	89601a	0	82.3	5.1
	74A	0	100	6.7
3	89601a	3.6	92.1	6.3
	74A	0.6	100	11.5
4	89601a	0	85.0	7.9
	74A	0	98.5	13.2
5	89601a	0.6	83.3	5.1
	74A	0	98.3	9.3
6	89601a	1.9	87.5	7.3
	74A	0.3	92.9	8.6
7	89601a	5.4	91.7	13.6
	74A	0	94.1	10.7
8	89601a	17.2	93.0	22.3
	74A	4.6	94.3	14.4

10 per cent of the total invertase activity is secreted into the medium until about the seventh day, after which larger amounts appear. A higher percentage of invertase and amylase is extracellular in 74A as compared with 89601a, although the reverse is true for trehalase.

These data were extended in a time-course experiment like that described in Methods during which samples were obtained at intervals of 2 hrs. At the end of 136 hrs of growth in this system, there is more than 20-fold more invertase in the medium in which strain 89601a is growing than in that of 74A. Despite the large disparity in the extracellular enzyme produced by these two strains, the glucose in the medium disappears at almost identical rates in both and the enzyme does not appear in either until the carbon source has disappeared.

Fig. 1 reveals that derepression occurs at about the same time in both strains, suggesting that they are equally sensitive to catabolite repression.

The question of whether repressible hydrolases are simultaneously or separately derepressed was addressed by analyzing amylase and invertase activity in the medium in which strain 89601a was grown in the preceding experiment. As can be seen in Fig. 2, these extracellular

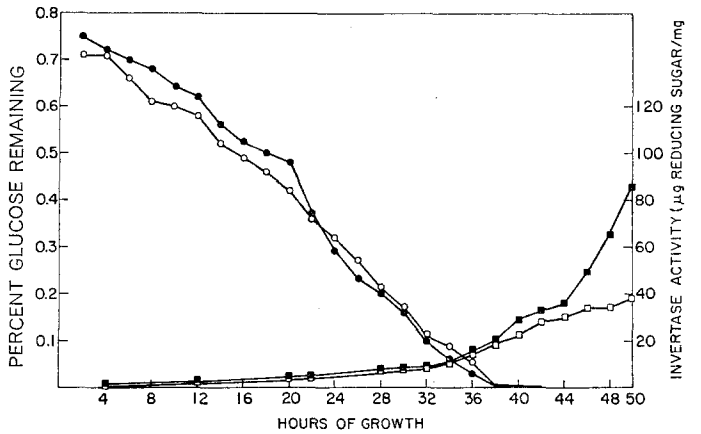

Fig. 1. Time-course of secretion of extracellular invertase by strains 89601a and 74A of *Neurospora crassa*. These strains were grown in continuous cultures as described in the text and aliquots were removed periodically from the medium and analyzed for invertase activity and residual glucose. Invertase activity: strain 74A (□); strain 89601a (■). Glucose remaining in the medium: strain 74A (○); strain 89601a (●). Enzyme activity was calculated from the reducing sugar released and was based upon mg of mycelium used, on a dry-weight basis

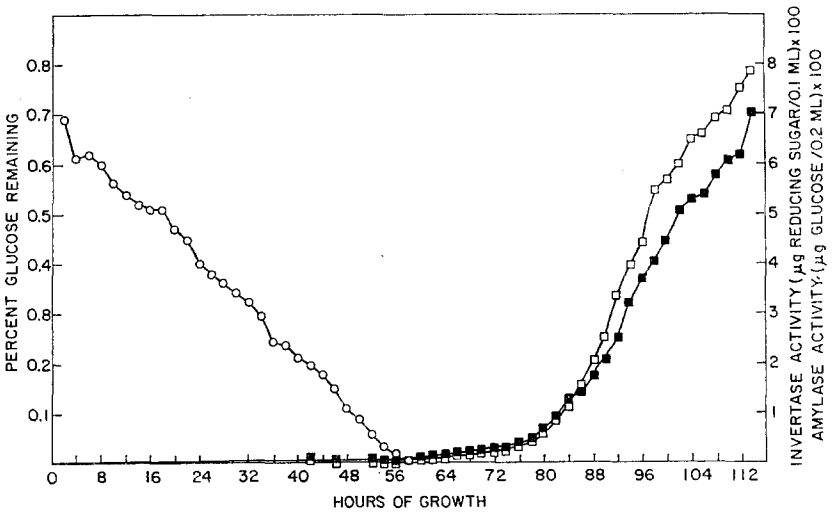

Fig. 2. Comparison of extracellular invertase and amylase activities formed by strain 89601a grown in continuous culture as described for Fig. 1. Invertase activity (■); amylase activity (□); glucose remaining in the medium (○). Enzyme activity was calculated on the basis of reducing sugar released and was based upon the volume of enzyme extract used

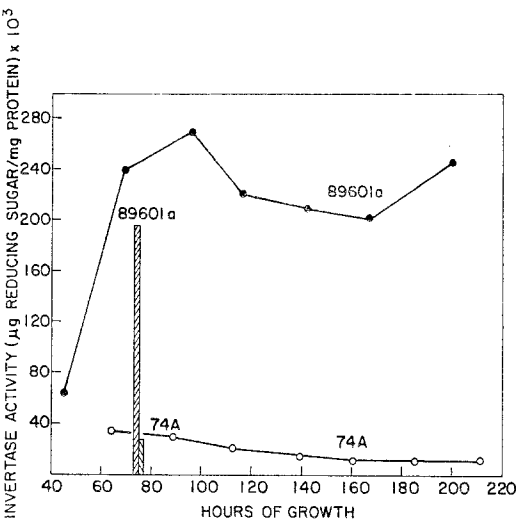

Fig. 3. Intracellular invertase activity in strains 74A and 89601a grown on cellulose as the sole carbon source. Activity: strain 74A (o); strain 89601a (•). The histograms indicate the activity of cultures of these strains which had been grown on Vogel's medium for 3 days at 20° C and transferred to a similar but carbon-free medium for 72 hrs

enzymes seem to be simultaneously derepressed after the disappearance of glucose from the medium. Similar experiments performed with the standard strain lead to the same conclusion.

A further test of whether the sensitivity of strain 89601a to catabolite repression is altered was applied. Cultures of strains 89601a and 74A were grown on Vogel's medium, with a layer of uncoated cellophane as the carbon source. This medium was chosen to obtain the slow release of a more or less constant level of glucose with the expectation that the culture would be continuously derepressed. In fact, as the data in Fig. 3 reveal, this condition almost is achieved in the case of invertase in both strains. Thus, when the activity on cellulose is compared with that after 72 hrs on a medium lacking carbohydrate, the former is consistently higher in the case of strain 89601a after about 100 hrs of growth. This effect is less evident in the case of strain 74A in which invertase activity on cellulose falls below that on the carbon-free medium after about 100 hrs of growth. Trehalase activity in both strains on cellulose is lower than that found in cultures on the carbon-free medium (Fig. 4). In the case of strain 74A the activity on cellulose only approaches the other after 200 hrs of growth.

This experiment lent itself to a test of whether the mechanism of enzyme induction is affected in the hyperderepressed strain. Thus,

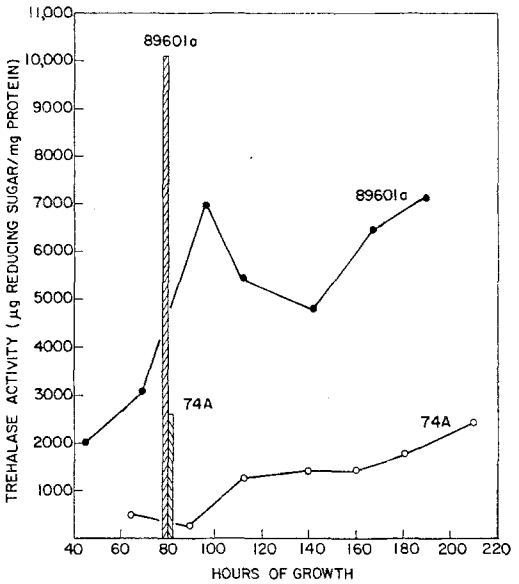

Fig. 4. Intracellular trehalase activity in strains 74A and 89601a grown on cellulose as the sole carbon source. Activity: strain 74A (o); strain 89601a (•). The histograms indicate the activity of cultures of these strains which had been grown on Vogel's medium for 3 days at 20°C and transferred to a similar, but carbon-free, medium for 72 hrs

Eberhart and Beck (1973) have shown that B-glucosidase is induced by cellobiose in *N. crassa* so it was reasonable to expect that the cellulose medium described above might serve as a source of cellobiose for induction after its degradation had begun. Indeed, as can be seen in Fig. 5, over three-fold more "cellobiase" is formed by strain 89601a on cellulose than on the carbon-free medium and more than five-fold more in the case of strain 74A, although the lag before induction is more pronounced in the latter case. It should be noted that although Eberhart and Beck have found two B-glucosidases in *Neurospora*, one of them, their "cellobiase" *sensu strictu*, has more than 20-fold greater affinity for cellobiose than the other, an aryl-B-glucosidase. In any event, the idea that cellobiose induces enzyme(s) which use it as a substrate is still valid.

Strains 89601a and 74A were crossed and randomly selected ascospores were isolated and cultures from these grown on Vogel's medium containing glucose. These cultures were harvested after 4 days and amylase, invertase, trehalase and alkaline phosphate activities were determined. In a separate experiment the ability of these segregants to grow without inositol also was tested. These data are presented in

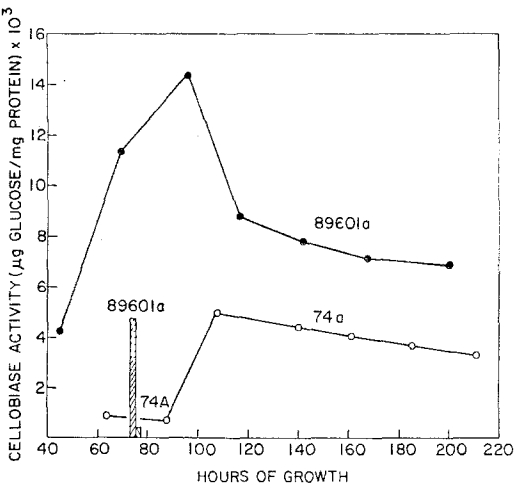

Fig. 5. Extracellular cellobiase activity in strains 74A and 89601a grown on cellulose as the sole carbon source. Activity: strain 74A (o); strain 89601a (•). The histograms indicate the activity of cultures of these strains which had been grown on Vogel's medium for 3 days at 20°C and transferred to a similar, but carbon-free, medium for 72 hrs

Table 3 in which the segregants are listed in the decreasing order of their amylase activity. In fact, this order holds in a general way for invertase and trehalase activities as well although exceptions can be noted. On the other hand, there seems to be no similar correlation with alkaline phosphatase activity. Table 3 also reveals that auxotrophy with respect to inositol is randomly distributed among the segregants.

When histograms of frequency against enzyme activity are examined (Fig. 6a—d), it can be seen that almost equal numbers of segregants with high amylase activity ($>$ 2,500 specific activity), and with low ($<$ 2,500 specific activity) are found and those with high activity appear to follow a Gaussian distribution (Fig. 6a). The amount of invertase activity also seems to segregate as a single gene (Fig. 6b) although this possibility is less clear than in the case of amylase. The data for trehalase are even less clear (Fig. 6c) and there is no pattern of segregation discernible at all in the case of alkaline phosphatase activity (Fig. 6d).

In order to determine how widely the allele for hyperderepression is distributed, the repressed and derepressed activities of the intracellular and extracellular glycosidases and alkaline phosphatase in several wild-type strains were compared with those in strain 89601a. All cultures were grown for 3 days at 21°C on Vogel's medium with glucose and

Table 3. Activity of some enzymes in segregants from a cross between strains 89601a and 74A. The segregation of their ability to grow without a supplement of inositol also is shown

Segregant	Enzyme activity				Growth without inositol
	Amylase	Invertase	Trehalase	Phosphatase	
88	16 380	29 673	1 310	265	−
83	15 720	51 998	2 530	345	+
89	14 113	25 817	350	214	−
95	14 013	31 749	1 650	303	+
80	13 168	38 711	1 520	437	+
52	13 104	23 778	170	306	−
94	12 108	28 577	1 080	257	−
56	10 896	21 842	1 530	261	−
58	10 544	30 610	1 620	329	+
65	10 286	28 357	1 530	289	+
49	9 907	22 230	1 980	322	−
85	9 281	22 735	1 170	253	+
87	9 073	21 003	1 230	233	−
61	8 904	24 968	940	213	+
54	8 657	17 210	1 260	272	−
92	8 455	21 467	710	243	−
91	7 304	22 156	1 640	295	+
86	7 000	15 800	280	158	−
78	6 566	16 098	830	228	−
57	6 043	12 097	890	283	+
96	5 616	4 073	430	199	+
64	5 007	12 576	230	165	−
66	4 237	6 871	610	237	+
51	4 042	12 117	240	49	−
70	3 598	10 414	140		+
90	2 938	10 634	700	318	+
50	2 094	5 987	1 270	181	+
93	1 901	7 489	600	157	+
63	1 859	9 158	710	248	+
68	1 847	5 827	590	254	+
60	1 735	3 400	1 170	200	−
72	1 638	5 388	530	209	+
55	1 607	5 861	680	166	+
53	1 601	5 208	930	132	−
62	1 601	4 785	560	199	−
81	1 569	4 743	700	195	+
84	1 565	5 100	940	241	−
69	1 559	5 967	610	173	+
67	1 503	4 733	750	255	−
77	1 430	4 677	700	266	−
82	1 411	3 367	470	284	−
75	1 374	3 311	610	191	−
76	1 333	4 648	650	159	−
74	1 333	4 446	510	188	−

Table 3 (continued)

Segregant	Enzyme activity				Growth without inositol
	Amylase	Invertase	Trehalase	Phosphatase	
59	1 310	6 814	720	118	—
71	1 250	7 249	790	184	—
79	1 177	3 991	480	224	+
48	1 135	3 600	670	40	—
73	1 700	5 049	170	44	—
74A	1 220	6 986	431	47	+
89601a	13 540	32 389	1 423	38	—

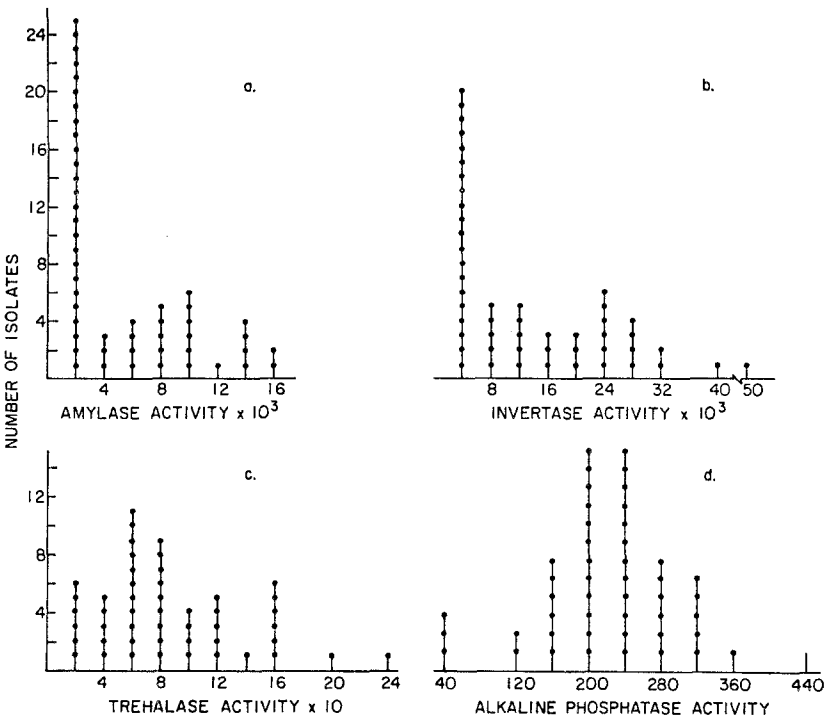

Fig. 6 a—d. Histograms showing distribution of enzyme activities in segregants from a cross between strains 74A and 89601a. (a) amylase activity; (b) invertase activity; (c) trehalase activity; (d) alkaline phosphatase

transferred to a medium without a carbon source for 72 hrs and harvested. A control set (repressed) was grown on 4% glucose for 50 hrs and harvested. It is clear from the data in Table 4 that strain 89601a is unique in its ability to hyperderepress amylase and cellobiase activity

Table 4. Repressed and derepressed enzyme levels in various wild-type strains of *Neurospora crassa* and in strain 89601 a

Enzyme	State/Time	Strain							
		Chilton	Yale	Lindegren	Lein	Abbott	Emerson	74 A	89601 a
Glucoamylase	rep./50 hrs	132	45	26	27	17	24	26	25
	derep./24 hrs	83	78	221	75	48	271	90	3 000
	derep./72 hrs	542	160	577	763	405	963	422	19 500
Trehalase	rep./50 hrs	21	8	19	8	11	29	10	21
	derep./24 hrs	1 458	406	4 204	846	325	1 667	820	1 267
	derep./12 hrs	8 750	1 745	4 487	2 813	1 741	3 186	2 611	10 125
Invertase	rep./50 hrs	1 143	1 568	817	1 939	1 307	1 952	521	2 214
	derep./24 hrs	21 500	13 086	12 295	37 500	11 111	43 750	20 902	70 667
	derep./72 hrs	71 875	9 906	16 026	59 375	12 500	56 875	28 333	195 000
Alkaline phosphatase	rep./50 hrs	0.80	0.46	0.60	0.46	0.52	0.60	0.56	0.50
	derep./24 hrs	1.306	0.594	1.311	0.833	0.667	1.042	0.754	0.907
	derep./72 hrs	10.333	1.500	5.462	3.000	3.000	2.850	2.633	5.250
Cellobiose	rep./50 hrs	54	123	63	46	30	97	62	39
	derep./24 hrs	81	125	213	417	67	333	213	440
	derep./72 hrs	542	208	923	400	493	425	400	4 750

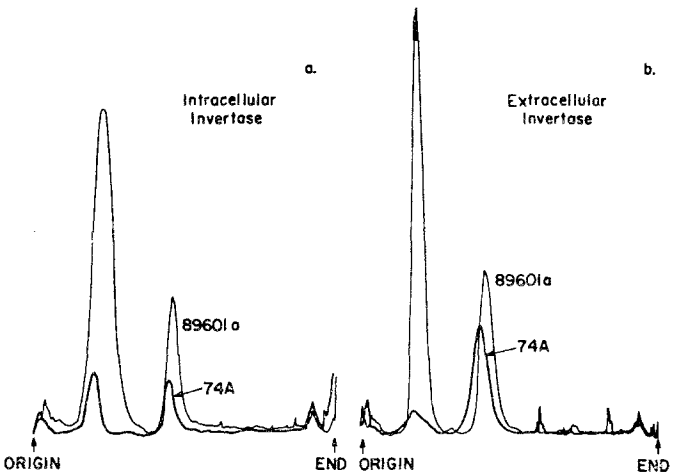

Fig. 7 a and b. Tracings of acrylamide gels run with extracts and medium from cultures of strains 74A and 89601a grown for 5 days at 20° C in shaken culture. The gels were stained for invertase activity and a Joyce-Loebl densitometer used to make the traces. Trace (a) is for intracellular and mural invertase and trace (b) is for extracellular invertase

among the strains tested. Although invertase and trehalase also show the highest activities in strain 89601a, there is a wider range of activities distributed through the wild-type strains investigated. On the other hand the Chilton strain produces almost twice as much alkaline phosphatase as strain 89601a and the Lindegren strain shows a little more activity than the latter.

Two isozymes of invertase have been described from *Neurospora crassa* by Eilers *et al.* (1964) and Metzenberg (1964). According to the latter, the more slowly migrating of these is a multimer, or "heavy" form of the enzyme, whereas the faster migrating isozyme is a subunit of the other and is the "light" form. Consequently, it was of interest to determine whether the hyperderepression gene affects the types of isozymes formed and their secretion. Thus, cultures of strains 89601a and 74A were grown for 4 days at 20° C in shaken cultures and the extracellular invertase and that of the cell, which includes the mural component as well as the intracellular, were isolated separately and run on acrylamide gels as described in "Methods". Afterthe appearance of the bands and their stabilization, they were scanned with a Joyce-Loeble densitometer. The traces for the intracellular and extracellular enzymes from each strain were recorded on the same graph so that they could be compared, as in Fig. 7a and b. What is revealed by these traces

Table 5. Activity of isozymes of *Neurospora crassa* in the intracellular and mural, and extracellular fractions. Activities are in grams and were obtained by weighing the areas under the curves in Fig.7 a and b. Each datum is the average of 4 separate weighings

Strain	Intracellular and mural			Extracellular		
	"Heavy"	"Light"	Ratio of "Heavy": "Light"	"Heavy"	"Light"	Ratio of "Heavy": "Light"
74A	0.0068	0.0056	1.21	0.0034	0.0156	0.22
89601a	0.0603	0.0146	4.13	0.0423	0.0221	1.92

is that strain 89601a forms much more of the heavy isozyme than of the light one, both intra- and extracellularly. (It should be pointed out that although the traces for a particular strain are roughly comparable on a quantitative basis, those for different strains cannot be compared in this way because of differences in the amount of protein run in the gels.) On the other hand, strain 74A produces more of the light isozyme extracellularly whereas there is a slight predominance of the heavy one within the cell. These relationships are quantified in Table 5.

Discussion

Analysis of segregants from the cross between the hyperderepressed strain used (89601a) and a wild-type strain suggests that there is a single gene difference in this trait. Although the evidence is clearest when amylase activity is measured, a similar segregation pattern is suggested for invertase and, perhaps, trehalase. On the other hand, phosphatase activity is not affected by this gene. Furthermore, modifiers exist which regulate the extent of hyperderepression because a Gaussian distribution is found over a wide range of hyperderepressed activities (Fig. 6a and b).

Seven wild-type strains have been shown not to be hyperderepressed for amylase, invertase, and cellobiase activities so the gene appears not to be widely distributed through the usual stocks of *N. crassa* (Table 4). This is true of trehalase as well but one strain (Chilton) shows almost as much activity as strain 89601a, and the differences among strains are not as great as with the other enzymes. Thus, there appear to be other, and more specific, genes which regulate the amount of derepression of glycosidases in *Neurospora*.

That the action of the hyperderepression gene cannot be explained as being due to diminished sensitivity to catabolite repression is shown by the fact that synthesis of amylase begins at roughly the same point

in glucose depletion in both the hyperderepressed and normal strains (Fig. 2 and Table 2). In addition, conditions which lead to the constant provision of low levels of a carbon source, such as growth on cellulose, cause derepression of invertase and trehalase to the degree to be expected in both strains (Figs. 3 and 4). Further support for this argument is provided by a comparison of the amount of derepression of invertase and trehalase when grown on cellulose. Trehalase is more sensitive to catabolite repression than is invertase because the steady-state level of glucose formed on cellulose represses the former more than the latter. However, what is important to observe is that strains 89601a and 74A behave similarly in this regard, thereby corroborating the conclusion that the response to catabolite repression is not different in the two strains. A similar conclusion can be made about induced enzyme biosynthesis for the data in Fig. 5 suggest that cellobiase is induced in both strains and, in fact, to a proportionately greater extent in the normal strain.

The increase in enzyme activity due to the activity of the allele for hyperderepression is in the order, amylase-cellobiase $>$ invertase $>$ trehalase (Table 1). Thus, the gene is pleiotropic in its effect and polarity is demonstrated as well. The order of hyperderepression is paralleled by the order of the clarity of segregation patterns in the genetic studies (Fig. 6a—c) and by the uniqueness of the allele in strain 89601a in affecting enzyme activity (Table 4). The fact that this order also holds for the proportion of extracellular activity secreted by the several enzymes studied (Table 2) suggests that the action of the gene may be at the cell surface.

This conjecture is supported by the observation that hyperderepressed strains secrete more of the "heavy" invertase extracellularly (Fig. 7). That molecular sieving by the cell wall may determine the amount of the "heavy" isozyme secreted was suggested by Trevithick and Metzenberg (1966) and the fact that the wall is the major restraint upon the release of this enzyme is demonstrated by the work of Bigger *et al.* (1972) with "slime", a mutant lacking a cell wall.

In fact, Gratzner (1972) postulates that the gene for hyperderepression is associated with changes in the cell wall, based upon chemical analyses, and Murayana and Ishikawa (1973) invoke similar changes to explain the effect of a gene which modulates amylase activity in a *Neurospora* mutant. However, it is difficult to explain, if the wall controls synthesis, why *slime* in the experiments of Bigger *et al.* (1972), in the absence of any wall, forms only as much enzyme as normally derepressed strains. Consequently, removal of the wall, by *itself*, cannot explain hyperderepression and we suggest that membranes alone, or in conjunction with the wall, are the locus of this control. Thus, Yoneda *et al.* (1973) have shown, in a mutant of *Bacillus subtilis*, that markedly increased production of amylase and protease is accompanied by a qualitative difference in a membrane component. Further-

more, certain vesicular membranes have been found to occur in yeast only during the derepression of extracellular invertase, an observation supported by the earlier work of Calonge *et al.* (1969) with pectinase from another fungus, *Sclerotinia fructigina*. Also, the increase in cellulase activity obtained by treatment of *Trichoderma viride* with surfactants (Reese and Maguire, 1971), and the similar data of Eberhart and Beck (1970) with cellobiase from *Neurospora*, support the role of membranes in hyperderepression.

The data in Table 5 bear upon the suggestion by Chang (1971) that enzymes of higher molecular weight are more likely to be trapped in the mural compartment by rigidification of the surface than are those of smaller molecular weight. Thus, our data reveal that in both of the strains used the proportion of the large molecular weight enzyme is greater in the mural and intracellular fraction than in the extracellular one. That this difference is greater in the case of strain 74A might argue that the surface of strain 89601a has been altered.

Should differences between the cell surfaces of strain 89601 and wild-type strains be found, the suggestion by Gingell (1973) that the aggregation of glycoproteins can cause changes in permeability is worth examining. Thus, phenomena like exocytosis and pinocytosis might be affected by the aggregation of such glycoproteins, many of which are secreted as extracellular enzymes. For example, suppose that glycoproteins penetrate lipid bilayers, as Bretscher (1971) has shown, then aggregation could lead to increased permeability of the membrane to the glycoproteins themselves, so that the more of these proteins that penetrate, the more are secreted. Therefore, a mutation in membranes could lead to the increased secretion of hydrolases, and to hyperderepression and increased synthesis, if feedback control over their synthesis is exerted by the intracellular system.

On the other hand, alternative explanations have been proposed for the pleiotropic mutants of *Serratia marcescens* in which there is an increase in extracellular nuclease, marcessin A and lipase activities, in addition to other effects (Winkler and Timmis, 1973). In this case, the other possibilities mentioned that might serve to explain hyperderepression in Neurospora include the proportion of higher levels of a regulatory substance of general importance, like cyclic 3′, 5′-adenosine monophosphate (cAMP). Support for this hypothesis derives from work which shows that cAMP stimulates the synthesis of colicin E (Nakazawa and Tamada, 1972) and chloramphenicol transferase (Harwood and Smith, 1971). However, inasmuch as cAMP may act upon membranes, this hypothesis is not incompatible with that we advance.

These data also bear upon the mechanism through which extracellular enzymes are secreted. Thus, although the synthesis of intracellular invertase precedes that of amylase (Table 1) the extracellular

enzymes are produced simultaneously. This suggests a common secretory mechanism may exist for the two enzymes, perhaps one based upon the exocytosis of vesicles, as discussed above.

These data generally support those of Gratzner and Sheehan (1969) and of Gratzner and Fass (1968) and suggest that we have worked with the same gene, labeled by those authors as *exo-1*. However, beyond our finding that the gene we have studied is not linked to *inos* (linkage group V), we have no evidence for its location. Despite the agreement with the work of Gratzner and co-workers in other respects we have found only a very slight effect of *exo-1* on trehalase. In fact, although Gratzner (1972) mentions that trehalase is hyperderepressed by the gene in his later work, his original data do not show much effect.

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Dr. A. S. Sussman
Department of Botany
University of Michigan
Ann Arbor, Michigan 48104
USA