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INFLUENCE OF THE CARBON SOURCE ON GLYCEROL KINASE ACTIVITY IN *NEUROSPORA CRASSA*

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Summary

The level of glycerol kinase activity in *Neurospora crassa* was shown to change in response to resuspension of sucrose-grown mycelia in fresh medium containing a new carbon source the magnitude of the change depended on the new carbon source provided Certain carbon sources, such as glucose and fructose, inhibited the small increase that occurred in the absence of any carbon source Others, and in particular deoxyribose, galactose, glycerol and ribose, greatly enhanced this increase The activity induced by deoxyribose and galactose had the same stability, both in vivo and in vitro, as that induced by glycerol, and as that induced by incubation of *Neurospora* cultures at low temperatures The inhibitory carbon sources, such as glucose and fructose, also restricted the increases induced by deoxyribose, galactose and glycerol they had more effect on the increases induced by glycerol and deoxyribose than on that induced by galactose The increase in activity that occurs at low temperature was also inhibited by glucose and sucrose

Introduction

Glycerol kinase (ATP glycerol-3-phosphotransferase, EC 2 7 1 30) catalyzes the stereospecific phosphorylation of glycerol, and in many microorganisms it is the first enzyme of the pathway of glycerol dissimilation [1] In *Neurospora crassa* the level of glycerol kinase activity increases when cultures are incubated in medium containing glycerol [2—4], and so it is probable that it performs the same function in this organism A study of the control of the enzyme has indicated that glycerol kinase may, however, play some additional role in *Neu-*

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rospora High levels of activity are induced in the absence of exogenous glycerol when cultures are incubated at low temperatures [2] In addition, both this cold-induced activity and that induced by glycerol at the normal growth temperature (26° C) are subject to continuous rapid inactivation in vivo [5]. Thus the activity can be kept under tight control, and this suggests that the enzyme may have some regulatory function

Denor and Courtright [6] have recently suggested that glycerol is the only known effector of the glycerol kinase system at normal growth temperatures, and have concluded that the cold-induced increase must result from the generation of an internal inducer, probably glycerol 3-phosphate In this report evidence is presented to show that under certain conditions at 26° C the level of glycerol kinase is markedly affected by the carbon source, and that compounds other than glycerol can also induce high levels of activity. It is also demonstrated that glycerol kinase is particularly sensitive to carbon catabolite repression, since the increases in activity can be inhibited by a number of carbon sources The control of glycerol kinase in *Neurospora* is discussed in the light of these findings

Materials and Methods

Organism *N crassa* strain 74A (wild-type) was used in all experiments.

Growth conditions and extract preparation Cultures were grown in either 20- or 100-ml portions of Fries minimal medium [7] in 50- or 250-ml Erlenmeyer flasks, respectively. They were inoculated from a conidial suspension (to give approx. 2 10^4 conidia/ml) and grown initially in medium containing 2% (w/v) sucrose as sole carbon source for 48 h at 26° C, with the flasks shaken orbitally in air (100 rev /min). The mycelia were then filtered over a Buchner funnel, washed well with distilled water, and resuspended in the appropriate fresh medium Incubation was then continued as before. Aseptic procedures were used whenever a further incubation period of longer than 2 h was employed.

For low temperature incubation cultures were initially grown in sucrose medium for 48 h at 26° C and were then transferred to 4° C (with reciprocal shaking in water) without resuspension in fresh medium

Extracts were prepared as described previously [2] in 83 mM tris(hydroxymethyl)aminomethane buffer (pH 8.0) from whole 20-ml cultures or portions (10 ml) of 100-ml cultures.

Assays Glycerol kinase activity was assayed at 25° C by the radiochemical method described previously [2] Protein was assayed by the method of Lowry et al [8], using bovine serum albumin as standard Glycerol kinase activity is expressed as nmol of glycerol phosphate bound (to DE-81 filter) per h per mg of protein

Sources [1-^{14}C]Glycerol was supplied by the Radiochemical Centre (Amersham, U K) α-D-Talose was supplied by Koch Light Laboratories Ltd (Colnbrook, U K) Adonitol, cyclohexamide, 2-deoxy-D-ribose (deoxyribose), D-erythrose, D-lyxose, D-tagatose, D-xylitol and L-xylose were supplied by Sigma London Chemical Co (Kingston-upon-Thames, U K). All other chemicals were obtained from B D H Chemicals Ltd. (Poole, U K.).

Results

Effect of changes of carbon source on glycerol kinase activity

When mycelia of *N crassa* that have been pre-grown in sucrose medium are transferred to fresh medium lacking a carbon source there is a small and transient increase in glycerol kinase activity [2] The effect on glycerol kinase activity of adding a number of sugars and related compounds to the fresh medium is shown in Table I During the 2-h period studied there was no significant change in the dry weight or protein content of the mycelia, and so in all cases changes in specific activity reflect changes in the actual level of intracellular glycerol kinase activity

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON THE LEVEL OF GLYCEROL KINASE ACTIVITY IN SUCROSE-GROWN MYCELIA

Mycelia from portions (20 ml) of 100-ml sucrose-grown cultures were resuspended in 20 ml of fresh medium without sucrose, but containing the compound indicated at a concentration of 5 mg/ml unless otherwise specified Extracts were prepared after 2 h of incubation The relative increase is the observed increase in specific activity from the initial level (3 5 units) relative to that observed in a culture to which no addition was made

Addition	Specific activity	Relative increase
None	14 4	1 0
Acetate (sodium salt)	12 0	0 8
Ethanediol	29 6	2 4
DL-Glyceraldehyde	43 6	3 6
1,3-Dihydroxyacetone	25 4	2 0
Glycerol	80 0	6 9
Erythrose	33 6	2 7
Erythritol	62 0	5 2
D-Arabinose	36 0	2 9
D-Lyxose	35 9	2 9
D-Ribose	88 0	7 6
D-Xylose	31 8	2 6
L-Arabinose	25 5	2 0
L-Xylose	24 5	1 9
Adonitol	6 2	0 3
L-Arabitol	12 0	0 8
D-Xylitol	11 8	0 8
Deoxyribose (2 5 mg/ml)	142 0	12 4
D-Galactose	65 6	5 6
D-Glucose	3 5	0
D-Mannose	4 6	0 1
α-D-Talose	20 5	1 5
D-Fructose	8 0	0 4
L-Sorbose	58 8	5 0
D-Tagatose (1 5 mg/ml)	11 4	0 7
Mannitol	7 7	0 4
Sorbitol	8 8	0 5
L-Fucose (1 5 mg/ml)	11 3	0 7
L-Rhamnose (1 5 mg/ml)	29 3	2 4
Cellobiose	8 0	0 4
Sucrose	4 5	0 1

On the basis of its effect, a compound could be placed in one of three groups There were those (acetate, arabitol, tagatose etc.) that had little effect on the increase in activity after resuspension This lack of effect could have been due to an inability of the cells to take up these compounds, and not necessarily because they lacked an effect on the glycerol kinase system per se. A second group of compounds inhibited the increase, either partially or completely, and were considered to have a negative effect on the glycerol kinase system All those compounds that support rapid growth of *Neurospora*, such as glucose, fructose, mannose and sucrose (see ref 9), were inhibitory, but the effect was also observed with some poorer sources of carbon such as sorbitol and mannitol Compounds in the third group all gave rise to increases in activity greater than that observed with no carbon source.

The third group contained a wide variety of compounds Some, such as ethanediol, glyceraldehyde, dihydroxyacetone and erythritol, were structurally similar to glycerol, many others, such as galactose, sorbose and all the pentoses tested, did not resemble glycerol so closely. Although many of these compounds are poor sources of carbon for *Neurospora* [9,10], the increases in activity could not have been directly related to this, since the complete lack of carbon source resulted in a much smaller increase in activity.

After 2 h of incubation in medium containing deoxyribose or ribose, the levels of activity were greater even than that found after equivalant incubation in medium containing glycerol. This greater ability to induce high levels of glycerol kinase activity was confirmed when the time course of some of the changes in activity were followed (Fig 1) The magnitude of the increase was in all cases greater than that observed in the absence of a carbon source [2] With galactose and xylose there was a small increase (2-fold) in the mycelial protein

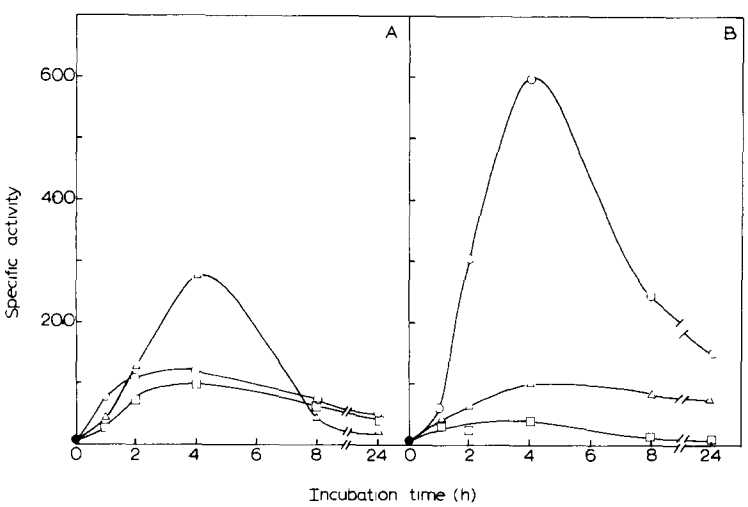

Fig 1 Time course of changes in glycerol kinase specific activity Mycelia from 100-ml sucrose-grown cultures were resuspended in 100 ml of fresh medium containing the carbon sources indicated at 20 mg/ml except for deoxyribose added at a concentration of 5 mg/ml During continued incubation, samples were removed and extracts prepared Symbols (A) ○———○, glycerol, □———□, DL-glyceraldehyde, △———△, D-ribose (B) ○———○, deoxyribose, □———□, D-xylose, △———△, D-galactose

level over the 24-h period, but no significant change with the other compounds In every case, however, the specific activity increased to reach a maximum approx. 4 h after resuspension, and then decreased The extent of this decrease was dependent on the carbon source The kinetics were similar, albeit over a shorter time period, to those observed for the increases in activity that take place at low temperatures [2] The level of activity achieved during incubation in the presence of deoxyribose was greater than that observed under any other conditions

Many of those compounds that caused an elevated level of glycerol kinase activity after resuspension of sucrose-grown mycelia gave rise to very low levels of activity when supplied as sole carbon sources throughout growth This was so whether the carbon source supported growth at a moderate rate (e g D-xylose) or at a very slow rate (e g D-ribose and deoxyribose) Of the compounds so tested, only D-galactose and glycerol allowed levels of activity significantly greater (30 and 70 specific activity units, respectively) than the basal level (1 5—4 units) characteristic of sucrose-grown cultures

Effects of cycloheximide

The increases in activity were all blocked by the addition of cycloheximide at the time of resuspension, and were therefore dependent on de novo protein synthesis [11]

The addition of cycloheximide to mycelia already incubating in medium containing galactose or deoxyribose resulted in an immediate and rapid loss of

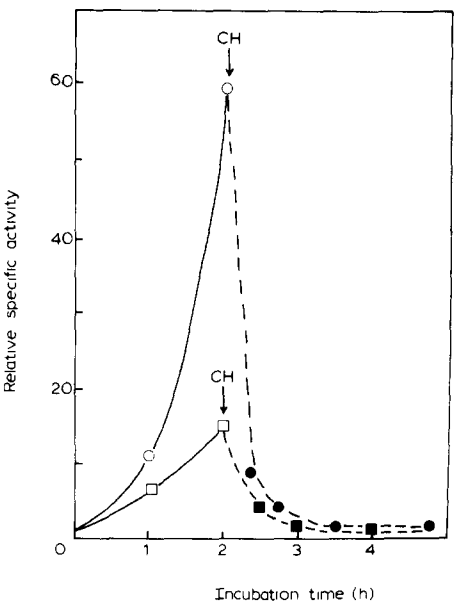

Fig 2 Effect of cycloheximide on glycerol kinase activity induced by deoxyribose and galactose Mycelia from 100-ml sucrose-grown cultures were resuspended in 100 ml of fresh medium containing 5 mg of deoxyribose (○———●) or 20 mg of galactose (□———■) per ml as sole carbon sources After 2 h of incubation 1 4 μg of cycloheximide (CH) were added per ml. Samples were removed at intervals and extracts prepared Glycerol kinase specific activity is given relative to that present at the time of resuspension (3 5 units, deoxyribose culture, 4 0 units, galactose culture)

activity (Fig. 2). As with the glycerol-induced activity and that induced by incubation at low temperatures [5], the half-life of the activity after the addition of the inhibitor was approx 15 min at 26° C

Thermostability of the induced activity

Since the property of rapid in vivo inactivation had been observed with every induced glycerol kinase activity from *Neurospora* to be tested (Fig 2 and ref 5), it was probable that the activities were due to the same enzyme This conclusion was supported by the results in Fig 3 which show that, in crude extracts, the activities induced by glycerol, deoxyribose, galactose and by incubation at 4° C had the same thermostability, with a half-life of 6 2 min at 67° C

Effects of additional carbon sources on increases in activity

When sucrose-grown mycelia were exposed to glycerol in the presence of other carbon sources the increase in glycerol kinase activity was affected (Table II) Incubation with a more readily utilised carbon source inhibited the increase sucrose and glucose were more effective than fructose In the absence of glycerol, D-galactose itself induced an increase in activity, and with glycerol it had an additive effect. Glycerol and deoxyribose together induced an increase in activity of the same order as that induced by deoxyribose alone

The increases in activity induced by galactose and deoxyribose were also sensitive to other carbon sources (Table II) Both were restricted by glucose and fructose, and as with the glycerol-induced increase glucose was a more

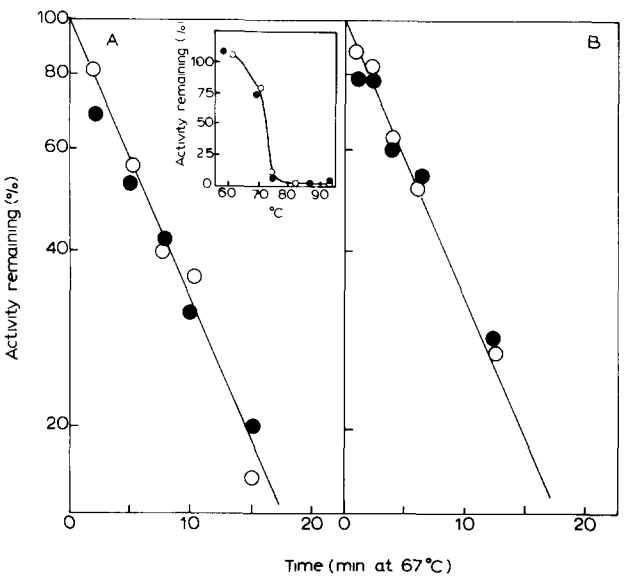

Fig 3 Heat inactivation of glycerol kinase activities Extracts were prepared from cultures incubated in glycerol (A, ○), galactose (B, ○) or deoxyribose (B, ●) medium for 2 h as described in Table I An additional extract was prepared from a 20-ml culture incubated for 48 h at 26° C and then for 48 h at 4° C (A, ●) Extracts were dialyzed overnight against 83 mM Tris buffer (pH 8 0), and were then incubated at 67° C and samples removed at intervals The glycerol kinase activity remaining in the sample was assayed The insert in (A) shows the effect of 2 min of incubation at different temperatures on the activity in extracts from the glycerol culture and the cold-incubated culture The activity remaining is given as a percentage of that present in the sample before incubation at elevated temperatures

TABLE II

EFFECT OF ADDITIONAL CARBON SOURCES ON THE INCREASE OF GLYCFROL KINASE ACTIVITY INDUCED BY GLYCEROL, GALACTOSE AND DEOXYRIBOSE

Mycelia from portions (20 ml) of 100-ml sucrose-grown cultures were resuspended in 20 ml of fresh medium without sucrose, but containing 20 mg of glycerol, 20 mg of galactose or 5 mg of deoxyribose per ml, together with the other carbon sources as specified (concentrations in mg/ml) Extracts were prepared after 2 h of incubation The relative increase is the observed increase in specific activity from the initial level (1 5 units) relative to that observed in a culture in which no additional carbon source was present

Additional carbon source	Specific activity			Relative increase		
	Glycerol	Galactose	Deoxyribose	Glycerol	Galactose	Deoxyribose
None	72 5	71 5	155	1 00	1 00	1 00
Sucrose (10 mg/ml)	2 4	—	—	0 01	—	—
D-Glucose (20 mg/ml)	10 0	33 6	15 1	0 12	0 46	0 09
D-Fructose (20 mg/ml)	25 9	62 9	24 8	0 34	0 88	0 15
Glycerol (20 mg/ml)	—	136 0	136 5	—	1 92	0 87
D-Galactose (20 mg/ml)	136 0	—	154 0	1 90	—	0 99
Deoxyribose (5 mg/ml)	136 5	154 0	—	1 91	2 18	—

effective inhibitor than fructose However, the galactose-induced increase was significantly less sensitive to the presence of the other hexoses than was that induced by either glycerol or deoxyribose Incubation of mycelia in medium containing galactose and deoxyribose resulted in a similar increase to that observed with deoxyribose alone

The increase in glycerol kinase activity induced by incubation of mycelia at low temperatures [2] was also affected by the addition of carbon sources to the medium Table III shows that in these experiments glucose acted as an inhibitor in contrast to the findings of Denor and Courtright [6] who did not, however, give the concentration of glucose used, and incubated the mycelia at the initial higher temperature for a shorter period Fructose had very little effect, and in this respect the cold-induced increase more closely resembled the galactose-induced increase than the glycerol-induced increase However, neither glycerol nor galactose enhanced the cold-induced increase, glycerol, in fact, was

TABLE III

EFFECT OF ADDITION OF CARBON SOURCES ON THE COLD-INDUCED INCREASE IN GLYCEROL KINASE ACTIVITY

20-ml cultures were grown in sucrose medium for 48 h at 26°C and then for 48 h at 4°C At the time of the temperature change additions were made as indicated at a concentration of 10 mg/ml The relative increase is the observed increase in specific activity from the level in a culture harvested after 48 h at 26°C (3 5 units) relative to that observed in a culture to which no addition was made

Addition	Specific activity	Relative increase
None	83 6	1 00
D-Glucose	33 5	0 37
Glycerol	68 0	0 80
D-Fructose	75 0	0 89
D-Galactose	77 8	0 92

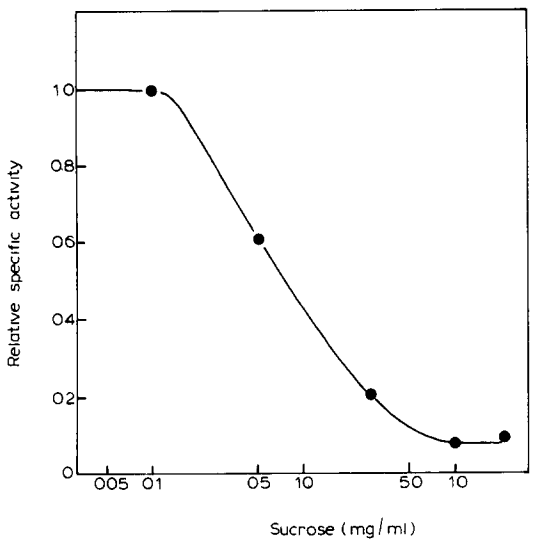

Fig 4 Inhibition of cold-induced increase of glycerol kinase activity by sucrose 20-ml cultures were incubated in sucrose medium for 48 h at 26°C and for 24 h at 4°C At the time of the change of temperature, more sucrose was added at the concentration shown The specific activity is given relative to that in a culture to which no extra sucrose was added (120 units)

slightly inhibitory. Addition of more sucrose to the medium at the time of the temperature change also restricted the increase over a wide range of concentrations (Fig 4), although some increase in activity was always observed.

Discussion

The data presented here clearly demonstrate that glycerol kinase activity in *N crassa* is sensitive to a change of carbon source, and that compounds other than glycerol can induce large increases in activity. Results in this (Fig 2) and a previous [5] report indicated that induced glycerol kinase was subject to continuous rapid inactivation Since the rate of inactivation (as measured by the loss of enzyme activity after the addition of cycloheximide) did not vary, and was not dependent on the composition of the medium, it was unlikely that any of the observed effects of carbon sources was due to an effect on inactivation Rather, both the increases in activity, and their inhibition, must have involved changes in the rate of production of glycerol kinase activity, almost certainly as a result of changes in the rate of de novo enzyme synthesis

In its response, albeit transient, to a change of carbon source glycerol kinase behaved in a similar manner to a number of other enzymes involved in the initial steps of carbon source utilization in *Neurospora* The activities of many disaccharidases are low in the presence of glucose, but are elevated by certain carbon sources that are not necessarily substrates for the particular enzyme [9,12–15].

The inhibitory effect of carbon sources such as glucose on the increase in activity that occurred after resuspension at 26°C (Tables I and II), and during incubation at 4°C (Table III and Fig. 4), demonstrated that glycerol kinase was

subject to carbon catabolite repression The inhibitory effects on the cold-induced increase provided an explanation for the observation that the level of activity attained during incubation at 4° C is dependent on the age of the culture at the time the incubation temperature is lowered the increase is much greater in cultures incubated for longer than 30 h at 26° C [10] During growth the level of sucrose (and glucose derived from it) will decrease, and so the inhibitory effect on the glycerol kinase system will diminish and allow greater cold-induced increases to take place It was unlikely that the inhibitory effects shown in Table II were due to an inhibition of the uptake of glycerol, galactose and deoxyribose· increases in activity that were not due to the presence of an effector in the medium were still subject to a 'glucose effect', both at 26° C (Table I) and 4° C (Table III and Fig 4) Moreover, sucrose prevents further production of glycerol kinase activity when the mycelia have already taken up glycerol for a period of 2 h [5]

A number of different compounds increased the level of glycerol kinase activity, and could thus be considered to be effectors of the system It is probable that more than one mechanism is involved There was evidence, for instance, that glycerol and galactose achieved their effects in different ways The effects were additive (Table II), and the increase in activity induced by galactose alone was much less sensitive to the 'glucose effect' than was that induced by glycerol (Table II) When supplied as sole carbon source, galactose elevates the activities of a number of other *Neurospora* enzymes whose activities are low with glucose as carbon source [12—15] Galactose probably releases enzyme synthesis from carbon catabolite repression, and elevated the glycerol kinase activity by overcoming repression that still occurred even in the absence of sucrose, this repression could have resulted from the endogenous metabolism that is known to occur in *Neurospora* [16] Glycerol was unlikely to have acted in the same way since it decreases the activities of other enzymes that are low in the presence of glucose but are elevated with galactose [17] Glycerol probably acted by a specific inducive mechanism, acting itself as the inducer, or, as in *Escherichia coli* [18,19], as a precursor of the true inducer

If an increase in glycerol kinase activity can be achieved both through a specific inducive mechanism and through general relief of carbon catabolite repression, then the wide range in the levels of activity achieved after resuspension (Table I) must have reflected the differing abilities of the carbon sources to affect both mechanisms It also indicates that the cold-induced increase may result from the partial release of glycerol kinase synthesis from catabolite repression, and not necessarily from the generation of an inducer Some sensitivity to carbon catabolite repression must be retained, however (Table III, Fig 4)

Since elevated glycerol kinase activities were not found in cultures grown on a single carbon source, except when this was glycerol or galactose, and, since the observed increases in activity were transient, it is possible that the enzyme was required for adaptation to the new growth conditions The inactivation of the enzyme would allow the rapid removal of the activity when adaptation was complete It has been observed [10] that during incubation of sucrose-grown mycelia in medium containing deoxyribose or ribose the increase in glycerol kinase activity is paralleled by an increase in the ability of the cells to incorporate glycerol carbon into macromolecules ($HClO_4$-insoluble material) Since the

mycelia gain this ability and the elevated glycerol kinase activity in the absence of exogenous glycerol, this could reflect a change on the endogenous metabolism of the organism. It is tempting to speculate that glycerol kinase may have a role in endogenous metabolism during adaptation periods

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