BIOSYNTHESIS OF INOSITOL BY INOSITOL-LESS MUTANTS OF *NEUROSPORA CRASSA*

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Abstract

Six inositol-less mutants of *N. crassa* were grown for 5 days on minimal medium containing [U-^{14}C]-D-glucose and *myo*-inositol (1·5 μg/ml). It was shown by a combination of electrophoresis, paper chromatography, and gas–liquid chromatography that in all cases a derivative of [^{14}C]-*myo*-inositol which behaves as an inositol monophosphate ester was present in the mycelium.

When the *myo*-inositol in the medium was increased to 6 μg/ml then the relative amount of [^{14}C]-*myo*-inositol derivative in the fungus was much reduced.

These results suggest that all the mutants examined are deficient in the single enzyme D-*myo*-inositol-1-phosphatase and that the activity of D-glucose-6-phosphate-cyclase is controlled by the level of free *myo*-inositol.

I. Introduction

The biosynthesis of *myo*-inositol from D-glucose 6-phosphate is well documented. The enzymes responsible for its synthesis in yeast (Chen and Charalampous 1966), *Neurospora crassa* (Pina and Tatum 1967), and rat testis (Eisenberg 1967) have been isolated and substantially purified. Nicotinamide adenine dinucleotide (NAD) and magnesium ions are known to be necessary cofactors. It is believed that the reaction involves an oxidation of D-glucose 6-phosphate at the 5-position followed by an aldol condensation to *scyllo*-inos-1-ose 2-phosphate which is then *cis*-hydrogenated to D-*myo*-inositol 1-phosphate (Chen and Charalampous 1966; Eisenberg 1967). During this reaction there is no incorporation of ^{3}H from NAD^{3}H into the product and the loss of ^{3}H from [5-^{3}H]glucose 6-phosphate is less than would be expected if the mechanism involved the formation of free intermediates (Barnett and Corina 1967). The cyclase reaction is thought, therefore, to be mediated by a single enzyme. Purification of the inositol synthetase system of *N. crassa* has resolved a magnesium-dependent phosphatase, D-*myo*-inositol-1-phosphatase, from the cyclase (Pina and Tatum 1967). It has thus been shown that two enzymes are involved in the conversion of D-glucose 6-phosphate to *myo*-inositol.

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During the course of recent attempts to find *myo*-inositol hexaorthophosphate (phytic acid) in microorganisms, inositol-less mutants of *N. crassa* were grown in the presence of either [^{14}C]-D-glucose or else [^{14}C]-*myo*-inositol and a search was made for phosphorylated derivatives of [^{14}C]-*myo*-inositol. This paper reports substantial chromatographic evidence for the presence of a compound behaving as [^{14}C]-*myo*-inositol monophosphate in the mycelium of six such mutants when grown in a medium low in inositol and containing [^{14}C]-D-glucose. These results are strong presumptive evidence that the biochemical lesion which renders these mutants absolutely dependent for growth upon exogenous inositol involves deletion of the enzyme D-*myo*-inositol-1-phosphatase and supports previous evidence (Partridge and Giles 1953) that such mutants are allelic. Results concerning the biosynthesis of phytic acid will be reported elsewhere.

II. Materials and Methods

(*a*) *Chemicals*

Uniformly labelled [^{14}C]-D-glucose (specific activity 2–4 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, England. Analytical reagents and glass-distilled water were used in all the experiments.

(*b*) *Organisms and Growth Conditions*

Inositol-less mutants of *N. crassa* (Nos. 37401, 64001, 89601, JH262, JH319, and JH5652) were obtained from Dartmouth College, Hanover, New Hampshire, U.S.A. and they were all shown to have an absolute requirement for inositol. These organisms were maintained on 2% (w/v) agar slopes of the minimal medium described by Nicholas and Nason (1954) containing glycerol (1% w/v), sucrose (1% w/v), and *myo*-inositol (6 μg/ml). The mutants were also regularly inoculated on to slopes devoid of inositol to check that there was no reversal to the wild type.

The [^{14}C]-D-glucose experiments were carried out in 20-ml test tubes in liquid minimal medium (4 ml) containing the required amount of inositol, glycerol, and sucrose. After inoculation from a stock agar slope, the cultures were incubated at 28°C for the required period.

(*c*) *Extraction of the Mycelium*

The mycelium was harvested and washed first in glucose solution (1% w/v) and then in water. It was then homogenized in perchloric acid (0·5 ml, 0·4N) for 3 min with an ultrasonic probe (20 kHz). The homogenate was centrifuged at 2000 *g* for 5 min and the clear supernatant fraction was stored at 2°C.

(*d*) *Incorporation of* [^{14}*C*]-D-*Glucose*

Aliquots (0·01 ml) of the culture medium, crude homogenate, and perchloric acid extract were plated on to stainless steel planchets and counted in a Beckman Low βII gas flow proportional counter.

(*e*) *High-voltage Paper Electrophoresis of the Extracts*

The apparatus and methods described by Tate (1968) were used. Aliquots (0·2 ml) of the extracts were subjected to electrophoresis on Whatman 3 MM chromatography paper (11 by 55 cm) wetted with oxalate buffer (0·1M, pH 1·5) at 40 V cm^{-1} for 45 min. The dried papers were sectioned and counted, and the position of phosphate bands was compared with that of suitable standards.

(*f*) *Chromatography of the Monophosphate Ester Band*

Areas of the electrophoretograms which contained [^{14}C]monophosphate esters were eluted twice with 1·5 ml 1N hydrochloric acid (95% recovery) using the centrifugation technique of

Hariharan, Poole, and Jones (1968). Carrier *myo*-inositol (75 μg) was added to each eluate which was sealed in a glass tube and heated overnight at 110°C.

The solutes were recovered and chromatographed on Whatman 3 MM chromatography paper in a descending manner (16 hr) with isopropanol–ammonia (80 : 20 v/v) at 25°C. Glucose and *myo*-inositol standards were used. Sections of the chromatograms were counted and then stained with alkaline silver nitrate (Anet and Reynolds 1954).

(*g*) *Determination of the Radioactivity of the Chromatograms*

The central strip (7 cm wide) of each chromatogram was cut transversely into sections 1·5 cm wide. Each section was counted in toluene scintillator [10 ml; 3 g 2,5-diphenyloxazole (PPO), 0·2 g 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) per litre] using a Parkard model 3375 liquid scintillation spectrometer. It was shown by the channel ratio method that the counting efficiency for both the electrophoretograms and paper chromatograms was approximately 84% and it did not vary appreciably. When counted the paper strips were placed in a large excess of toluene to remove the scintillator chemicals and then air-dried.

(*h*) *Gas–Liquid Chromatography and Counting of the* [^{14}C]*Inositol*

That area of the paper chromatogram which corresponded to the inositol markers was extracted with water and L-inositol (40 μg) was added to the eluate. The solids were recovered and acetylated in pyridine (0·5 ml) and acetic anhydride (0·5 ml). The mixture of acetates dissolved freely in 0·02 ml chloroform and 0·01 ml of this solution was separated in a Packard gas chromatograph using a 200 by 0·4 cm column containing 10% (w/v) ECNSS* (Applied Science Laboratories, Pennsylvania) on Embacel, 60–100 mesh, 200°C N_2 carrier, 25 ml/min. A stream-splitter in the effluent stream passed one-half to the hydrogen flame detector and the other half to an air-cooled glass capillary tube where any vapours condensed. Their passage into this tube was monitored by the detector and its associated chart recorder.

Immediately before the material to be counted appeared, the capillary tube was replaced with a fresh one which was removed as soon as efflux of the component had ceased. The contents of this capillary tube were then washed into a glass scintillation vial containing toluene scintillator (10 ml) and counted on the Packard liquid scintillation spectrometer.

III. Results

(*a*) *Synthesis of a* [^{14}*C*]*Inositol Derivative from* [^{14}*C*]-D-*Glucose*

In a preliminary experiment mutant 37401 was grown for 5 days on minimal medium containing glycerol (0·2%), sucrose (0·2%), *myo*-inositol (1·5 μg/ml), and [U-^{14}C]-D-glucose (5 μCi). Under these conditions there was a 6% incorporation of radioactivity of which 25% was acid-soluble. Chromatography of the hydrolysed monophosphate ester band isolated from the extract gave a single band of radioactivity just behind the *myo*-inositol markers and another one close to the glucose standards. When the products of the acid hydrolysis were de-ionized with Amberlite monobed resin MB1 before chromatography, then a single area of radioactivity corresponding exactly to *myo*-inositol was recorded whereas the area corresponding to glucose had disappeared.

Since the incorporation of labelled glucose in the preliminary experiment was small (6%), attempts were made to find a medium from which more [^{14}C]glucose was incorporated. A minimal medium containing glycerol (1%), glucose (0·02%), and *myo*-inositol (6 μg/ml) supported normal growth in all the mutants.

* An organosilicone polymer combining ethylene succinate with cyanoethylsilicones.

If the glucose was supplied as 20 μCi of low specific activity [^{14}C]-D-glucose (2–4 μCi/μmole) then after 5 days growth approximately 65% of the radioactivity

TABLE 1

RESULTS OBTAINED WHEN MUTANTS WERE GROWN IN MEDIUM CONTAINING HIGH (6·0 μg/ml) AND LOW (1·5 μg/ml) CONCENTRATIONS OF *myo*-INOSITOL

The mutants were grown in duplicate cultures on minimal medium plus *myo*-inositol and [^{14}C]-glucose. The mycelia were harvested after 5 days and extracted with acid from which the monophosphate esters were separated by electrophoresis. Hydrolysis of this fraction followed by paper chromatography (see Section II) showed radioactivity corresponding to both inositol and glucose

Mutant	Acid-soluble ^{14}C (%)	Activity (net counts/min) of:			Ratio of:	
		Mono-phosphate Ester Band*	Inositol Band†	Glucose Band†	Glucose to Total Mono-phosphate Esters minus Inositol	Inositol to Glucose Bands†
		Medium containing 6·0 μg/ml *myo*-inositol				
37401	8·8	20,596	540	4,812	0·241	0·112
	12·0	26,684	—	—	—	—
89601	14·0	34,028	992	5,808	0·175	0·146
	16·5	33,476	—	—	—	—
JH262	19·5	29,480	780	9,176	0·320	0·085
	19·5	29,904	—	—	—	—
JH319	14·0	15,956	228	3,196	0·203	0·071
	19·5	16,332	—	—	—	—
JH5652	18·8	20,308	144	4,412	0·218	0·033
	17·7	19,556	—	—	—	—
Means	16·0	24,632	536	5,480	0·231	0·089
S.D.	±3·7	±6,887	±359	±2,267	±0·055	±0·043
		Medium containing 1·5 μg/ml *myo*-inositol				
37401	13·1	11,112	1,499	2,432	0·253	0·62
	10·0	8,184	678	1,297	0·172	0·52
89601	11·3	5,517	763	1,351	0·285	0·56
	12·9	6,809	952	1,700	0·290	0·56
JH262	19·9	12,026	2,371	4,059	0·421	0·58
	20·6	17,873	3,130	5,016	0·339	0·63
JH319	16·5	9,214	1,055	2,106	0·258	0·50
	13·9	8,405	1,746	2,075	0·311	0·84
JH5652	12·9	8,934	944	3,788	0·474	0·25
	13·1	9,387	1,109	2,614	0·316	0·42
Means	14·4	9,747	1,424	2,643	0·312	0·54
S.D.	±3·5	±3,414	±788	±1,246	±0·086	±0·15

* Separated by electrophoreses. † From paper chromatography.

was incorporated into the mycelium and about 15% of this was acid-soluble (Table 1). This low-glucose medium was used for subsequent experiments.

(*b*) *Effect of Inositol Concentration in the Medium on the Synthesis of the* [^{14}C]*Inositol Derivative*

Inositol-less mutants were grown in a medium containing *myo*-inositol and the synthesis of the [^{14}C]inositol derivative from [^{14}C]-D-glucose was followed by the methods described above.

In all the electrophoretograms a peak of radioactivity coincident with a phosphate band which had the mobility of an inositol monophosphate and was well separated from inorganic phosphate and inositol diphosphates was observed. Hydrolysis of this band and paper chromatography of the residue gave in all cases two well-separated bands of radioactivity corresponding to *myo*-inositol and glucose. These results for five of the mutants are summarized in Table 1.

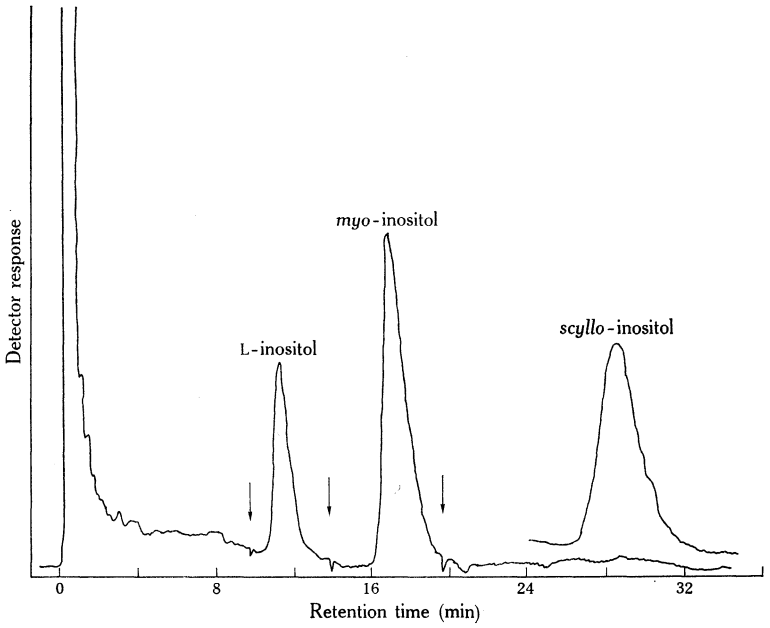


Fig. 1.—Gas–liquid chromatography of the hexa-acetates from the [^{14}C]inositol band (*myo*-inositol and L-inositol carriers added). The time at which *scyllo*-inositol appears is also shown. The vertical arrows indicate when the collection tube was changed (see Section II).

At the levels of *myo*-inositol used, namely 1·5 μg/ml and 6·0 μg/ml, this nutrient was growth-limiting after 5 days. This was reflected in the greater amount of mycelium, and also radioactivity in the extracts, from the mutants grown under the latter conditions. In order to compare the quantity of [^{14}C]inositol derivative produced in the two circumstances, the activity of the [^{14}C]inositol band has been expressed in each case as a fraction of the [^{14}C]glucose contained in the same electrophoretic fraction. It was shown that the quantity of such [^{14}C]glucose isolated from each mycelium relative to the total ^{14}C in the monophosphate ester band minus the [^{14}C]inositol was essentially constant. The mean value of the inositol : glucose ratio

was 0·548 when *myo*-inositol was present at 1·5 μg/ml in the growth medium but fell to 0·089 when the inositol level was increased to 6·0 μg/ml (Table 1).

(c) *Characterization of the* [^{14}C]*Inositol Moiety*

In additional experiments, four mutants were grown as before in a medium containing 1·5 μg/ml *myo*-inositol but were harvested after 3 days growth instead of 5 days. Investigation of the ^{14}C-labelled carbohydrates as described revealed that the mean inositol : glucose ratio was 0·20 under these less-limiting conditions. The inositol band was eluted with water and after adding L-inositol the hexa-acetates were prepared and chromatogrammed on a gas–liquid column (see Section II). A typical trace is shown in Figure 1 together with a *scyllo*-inositol standard which has been superimposed. The L-inositol and *myo*-inositol fractions were collected and counted. Calibration of the system with a standard solution of *myo*-inositol hexa-acetate enabled the percentage of inositol counted to be calculated. The results summarized in Table 2 indicate that a derivative of *myo*-inositol was present in the extracts of the mycelia.

TABLE 2

RESULTS OF GAS–LIQUID CHROMATOGRAPHY OF THE [^{14}C]INOSITOL FRACTION FROM PAPER CHROMATOGRAPHY

Mutants were grown in medium containing 1·5 μg/ml *myo*-inositol in the presence of [^{14}C]glucose and harvested after 3 days. The monophosphate esters were separated by electrophoresis from acid extracts of the mycelia and after acid hydrolysis these were further separated by paper chromatography. Gas–liquid chromatography of the acetylated [^{14}C]inositol band gave fractions which were collected and counted in toluene scintillator (see Section II)

Mutant	Acid-soluble ^{14}C (%)	Activity (net counts/min) of: Mono-phosphate Ester Band*	Inositol Band†	*myo*-Inositol Fraction‡	L-Inositol Fraction‡	Fraction [^{14}C]Inositol Band Counted (%)‡
37401	5·4	24,246	976	103±1	0	17·5
JH319	2·6	28,591	705	53±2	0	17·8
JH5652	2·8	22,517	732	48±2	0	15·5
64001	3·9	25,793	521	41±2	0	12·2

* Separated by electrophoresis. † From paper chromatography.

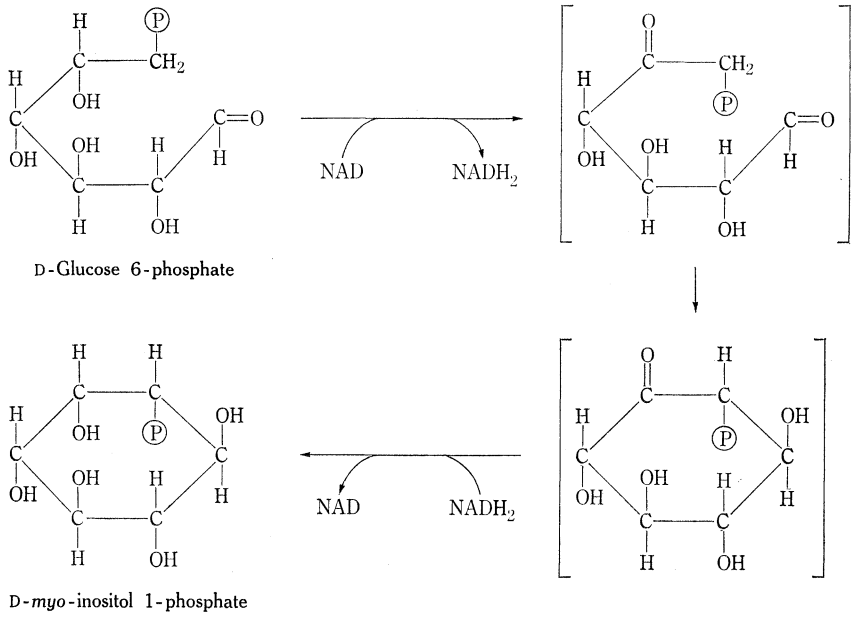
‡ From gas–liquid chromatography.

IV. DISCUSSION

The electrophoretic technique used to separate the inositol derivative from the acid extracts is not capable of resolving various hexose monophosphates such as glucose 6-phosphate, glucose 1-phosphate, fructose 1-phosphate, or inositol 1-phosphate but this group of compounds is well removed from inositol diphosphates which have a higher mobility (Tate, unpublished data). Any inositol isolated by electrophoresis as described would, therefore, not be polyphosphorylated.

The chromatographic technique used to fractionate the hydrolysate of the hexose monophosphate ester band separates inositols from reducing sugars but does not resolve the various isomers of inositol. Table 1, therefore, summarizes results which suggest the presence of a [^{14}C]inositol of indeterminate structure in the mycelium of the mutants and this conclusion is adequately substantiated by the results of the gas chromatographic analysis (Table 2). In addition, however, the latter technique can resolve *myo*-inositol from its L- and *scyllo*-isomers (Fig. 1) and it provides substantial evidence that the inositol derivative present in the mycelia which has the properties of an inositol monophosphate is a derivative of *myo*-inositol.

The biosynthesis of *myo*-inositol from D-glucose 6-phosphate in the wild-type of *N. crassa* is well documented and the two enzymes involved have been substantially purified (Pina and Tatum 1967). A cyclase dependent upon NAD first converts the substrate to D-*myo*-inositol 1-phosphate via a mechanism (Eisenberg 1967) compatible with the scheme shown in Figure 2. The first step is believed to be an



D-Glucose 6-phosphate

D-*myo*-inositol 1-phosphate

Fig. 2.—Conversion of D-glucose 6-phosphate to D-*myo*-inositol 1-phosphate by the cyclase enzyme.

oxidation at the 5-carbon position which results in a 1–6 aldol condensation to *scyllo*-inos-1-ose 2-phosphate. This inosose is then *cis*-hydrogenated to D-*myo*-inositol 1-phosphate. Evidence that a single enzyme is responsible is provided by the observation that there is no incorporation of ^{3}H from NAD^{3}H into the product and the loss of ^{3}H from [5-^{3}H]-D-glucose 6-phosphate is less than would be expected if the mechanism involved the formation of free intermediates. The product of this reaction is the optical enantiomorph of the inositol monophosphate found in lipids (Ballou and Pizer 1959) and has not been found elsewhere in nature. This product is not utilized further (Brown *et al.* 1960) before hydrolysis of the phosphate ester by a

specific phosphatase which is present in wild-type *N. crassa* and has been resolved from the cyclase (Pina and Tatum 1967). It is unlikely, therefore, that the *myo*-inositol derivative found in the mutants is other than the product of this reaction.

We conclude therefore that it is D-*myo*-inositol 1-phosphate, the substrate for this specific phosphatase, which accumulates in the mutants, all of which have an absolute requirement for the product, free *myo*-inositol. The lesion in the biosynthetic pathway of *myo*-inositol in all six mutants therefore involves deletion of the single enzyme D-*myo*-inositol-1-phosphatase. This conclusion substantiates the observations that all such mutants are allelic (Partridge and Giles 1953) and do not respond to inositol monophosphates (Angyal and Tate 1961).

Under conditions in which *myo*-inositol is not growth-limiting, e.g. 6 μg/ml at 5 days (Table 1) or 1·5 μg/ml at 3 days (Table 2) there is evidence for a smaller accumulation of [^{14}C]-*myo*-inositol monophosphate than under the more limiting condition of 1·5 μg/ml at 5 days (Table 1). It is likely therefore that the level of free inositol controls the activity of the inositol synthetase system. The activity of the cyclase, which we have shown to be present in the mutants, is known to be rate controlling in rat testes and kidney (Eisenberg 1967) and its activity may be regulated by a *myo*-inositol-mediated repression of the gene which codes for this enzyme. This circumstance would explain the failure of Pina and Tatum (1967) to isolate enzymes of the *myo*-inositol synthetic pathway from mutant 89601 grown on a medium containing 50 μg/ml *myo*-inositol.

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