INOSITOL FORMATION BY CYCLIZATION OF GLUCOSE CHAIN IN RAT TESTIS

Frank Eisenberg, Jr. and Arthur H. Bolden

National Institute of Arthritis and Metabolic Diseases
National Institutes of Health
Bethesda 14, Maryland

and Frank A. Loewus

Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service
United States Department of Agriculture, Albany, California

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A homogenate of rat testis capable of incorporating carbon from glucose-U-C^{14} into inositol has been described in a previous publication (Eisenberg and Bolden, 1963). In an attempt to differentiate[1] between a hexose moiety (Imai, 1963; Loewus and Kelly, 1962) and hexose fragments (Charalampous, 1957; Hauser and Finelli, 1963) as precursors of inositol, the conversion of specifically labeled glucose to inositol has now been studied in rat testis homogenate with the aid of a combined plant and chemical degradative system described previously (Loewus et al, 1962). Experimental evidence to be presented in this paper points unequivocally to a pathway of biosynthesis of inositol in the mammal involving cyclization of a six-carbon unit.

Biosynthesis of Inositol-C^{14}. Forty-five ml of 50% homogenate in isotonic KCl was prepared from decapsulated rat testis and added to 105 ml M/10 potassium phosphate buffer, pH 7.4. To each third of the suspension 5 mg inositol and 5 mg, resp., of glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} were added, followed by incubation for 2 hr with shaking in air.

[1] A soluble system which synthesizes inositol-C^{14} from glucose-U-C^{14} has been found in yeast (Chen and Charalampous, 1963).

Isolation of inositol as hexaacetate from the incubation mixtures after further addition of 13.5 mg carrier followed the original procedure (Eisenberg and Bolden, 1963) except for the acetylation step in which IR 120 resin (Christensen, 1962) replaced pyridine as catalyst. Inositol, 7-9 mg, liberated from the acetate was recrystallized to constant specific activity by precipitation from water with 9 vol methanol. Table I. Radiochemical purity of the final products was confirmed by paper chromatography in acetone:water (85:15) (Posternak et al, 1955) and in propanol:ethyl acetate:water (7:1:2) (Cerbulis, 1955). In a control experiment glucose-2-C^{14} failed to be incorporated into inositol when incubated with boiled homogenate.

Degradation[2] of Inositol-C^{14}. Inositol synthesized from each substrate was taken up in aqueous solution through the freshly severed stems of ripening strawberries. After incubation for 47 hr, during which time respiratory CO_2 was collected, the berries were macerated in cold EtOH. The insoluble residue, washed free of soluble radioactivity, was hydrolyzed for 36 hr with Pectinol 100D, a fungal pectinase. The clear supernatant fluid from this suspension was chromatographed on Dowex-1 formate with dilute formic acid. D-galacturonic acid, eluted as a single radioactive peak, was reduced to L-galactonic acid and degraded with $NaIO_4$ to CO_2 (C1 of inositol), HCOOH (C2-5), and HCHO (C6) (Seegmiller et al, 1955). For further localization of C^{14} in inositol from glucose-2-C^{14} a portion of galactonic acid was converted to galactonamide and degraded to HCOOH (C3-5) and HCHO (C6) (Loewus and Kelly, 1963).

Results. Table I shows that the relative specific activities of substrate glucose-C^{14} and product inositol-C^{14} are essentially identical.

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[2] The authors are indebted to Mr. Stanley Kelly for able assistance in this phase of the work.

Although this might be a fortuitous result, it suggests utilization of an intact hexose moiety in the formation of inositol in testis, a prediction supported by further evidence.

Table I

Conversion of Glucose-C^{14} to Inositol-C^{14} in Rat Testis Homogenate

Substrate	S. A. μC/mg	Rel. S.A.	Sp. Act.* Inositol-C^{14}			Rel. S.A.
			Stage 1	Stage 2	Stage 3	
Glucose-1-C^{14}	3.5	1	2480	2310	2410	1
Glucose-2-C^{14}	3.0	0.86	1978	2160	2170	0.90
Glucose-6-C^{14}	3.2	0.91	2142	2190	2080	0.86

*cpm/mg inositol, based on observed count rates of 200-300 cpm above background (20 cpm) determined on a 1% aliquot of total inositol crystallized (7-9 mg) at each stage of purification. Aliquot was spotted on Kieselgur, suspended in counting gel (Snyder and Stephens, 1962) and counted at 50% efficiency in a Packard Liquid Scintillation Spectrometer. Substrates, suitably diluted with glucose, were assayed for C^{14} in the same way.

Table II shows that strawberry produced 5-7 times as much $C^{14}O_2$ from inositol-C^{14} derived from glucose-6-C^{14} as from the other labeled inositols.

Table II

Conversion of Inositol-C^{14} to Galacturonic Acid-C^{14} of Pectin in Strawberry

Source of C^{14}	Inositol-C^{14}		Resp. $C^{14}O_2$		Galacturonic Acid-C^{14}	
	mg admin.	dpm*	dpm**	%	dpm**	%
Glucose-1-C^{14}	5.0	24,100	3,200	13.3	11,000	46
Glucose-2-C^{14}	5.0	21,700	2,100	9.7	5,500	25
Glucose-6-C^{14}	5.1	21,100	14,700	69.7	4,600	22

* counted at 50% efficiency and corrected to 100%.
** counted at 80% efficiency and corrected to 100%.

Since CO_2 originates from the carboxyl (C6) of a uronic acid (Loewus *et al*, 1958) which in turn was derived from C1 of inositol by oxidative cleavage at C1,6, the observed results are expected if inositol arises through cyclization of a six-carbon unit.

In accord with previous observations (Loewus and Kelly, 1962) inositol was found to be an active precursor of pectin; Table II shows that 22-46% of administered inositol was incorporated into the polysaccharide.

In Table III are recorded the results of degradation of galacturonic acid recovered from pectin synthesized from variously labeled inositols produced in rat testis homogenate. From the distribution of C^{14} in galactonate and galactonamide it can be seen that these inositols are specifically labeled to the extent of 80-96% and that C6 of inositol arises from C1 of glucose and *vice versa*. Fig. 1. Although the labeled

Table III

Distribution of Label in Inositol-C^{14} by Chemical Degradation of Galacturonic Acid-C^{14}

Inositol Carbon	Degradation Fragment	Source of C^{14}					
		glu.-1-C^{14}		glu.-2-C^{14}		glu.-6-C^{14}	
		S.A.*	%	S.A.*	%	S.A.*	%
1-6	D-galacturonate**	156	100	86	100	49	100
1	L-galactonate, C1	82	9	5	1	289	96
2-5	" C2-5	11	5	113	88	5	7
6	" C6	747	80	6	1	24	8
3-5	L-galactonamide, C3-5			163	95		
6	" C6			3	1		

* cpm/mg carbon, based on observed count rates of 2-700 cpm above background (60 cpm) on 1-3 mg of carbon as CO_2, counted at 80% efficiency in a gas phase (methane) proportional counting system.

** wet combustion.

carbon atom in inositol derived from glucose-2-C^{14} has not been identified, it is clearly confined to C3-5 and is presumed to be C5.

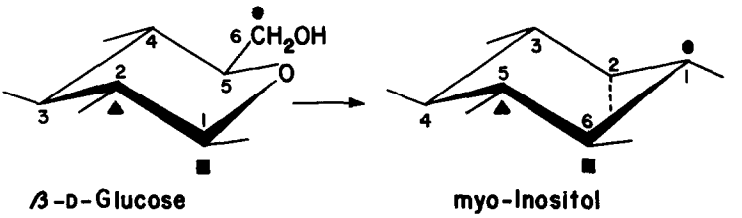

Fig. 1. Structural Relationship of β-D-Glucose and myo-Inositol. Symbols indicate labeling pattern of inositol synthesized enzymatically from specifically labeled glucose.

Discussion. The experiments described in this paper are the first in which the labeling pattern of inositol produced in vitro has been investigated. Through use of a cell-free preparation of actively synthesizing rat testis and a degradative system which reveals the location of label in inositol, it has been shown unequivocally that inositol is synthesized in the mammal by cyclization of a hexose unit.

From Fig. 1 it is clear that cyclization is a plausible mechanism (Fischer, 1944) requiring no conformational changes. A possible enzymatic role in this reaction involves stereospecific release of a hydrogen bound to C6 of glucose, or an intermediate, leading to the unique spatial orientation of C6 and C1 necessary for formation of inositol by ring closure. Studies to determine the actual enzymic mechanism of this isomerization are in progress.

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