BIOSYNTHESIS OF INOSITOL IN RAT TESTIS HOMOGENATE

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Received May 13, 1963

The observation of inositol formation in germ-free rats and mice (Freinkel and Dawson, 1961) has established unequivocally the existence of a mammalian pathway for the biosynthesis of this cyclitol. Until now studies designed to elucidate this pathway have utilized the intact cell either in the whole animal (Daughaday et al, 1955; Halliday and Anderson, 1955; Imai, 1963; Hauser and Finelli, 1963), tissue culture (Eagle et al, 1960), or tissue slices (Hauser and Finelli, 1963). Synthesis of inositol in intact non-mammalian cells has been observed in yeast (Charalampous, 1957), chick embryo (Daughaday et al, 1955), and plants (Loewus and Kelly, 1962). The present communication describes experiments in which for the first time a homogenate, prepared from rat testis, has been found active in the synthesis of myo-inositol from glucose in yields about 10 times greater than in mammalian cellular systems. Under the same experimental conditions homogenates of rat liver, kidney, brain, spleen, lung, and lactating mammary gland failed to incorporate glucose carbon into inositol.

In Vitro System. Rats weighing 145 - 300 gm were killed by decapitation. Organs were excised and homogenized in an equal volume of ice-cold isotonic KCl. A 3 ml aliquot (300 mg dry tissue) was added to 7.0 ml cold M/10 potassium phosphate buffer, pH 7.4, to which had been added 0.5 mg glucose-U-C^{14} and 1.0 mg inositol. The suspension was incubated for 2 hr with shaking in air and then stopped by heating for 15 min at 100°. The resulting coagulum was washed by centrifugation in

the Servall. To the combined supernatant fluids were added 100 mg glucose and carrier inositol (Table I).

Table I

Radiochemical Yields of Inositol-C^{14}
Synthesized by Homogenates of Rat Testis

Experiment	Glucose-U-C^{14} cpm*	Added ** In$(OAc)_6$ mg	Sp. Act. Isolated In $(OAc)_6$ cpm/mg	Incorp. %
1	5×10^5	7.4	170	0.25
2	5×10^5	12.0	240	0.58
3	5×10^5	12.0	214	0.51
4	5×10^5	12.0	289	0.69
5	1×10^6	12.0	479***	0.57
6	5×10^5	12.0	267.	0.64

* Spotted on Kieselgur and counted as described for inositol hexaacetate.
** Calc. from 432/180 x mg inositol added.
*** Reacetylated after regeneration from hexaacetate.

Removal of Glucose-C^{14}. The solution was shaken for 1 hr with 1 ml Br_2 and 500 mg $BaCO_3$ which were then removed by extraction with CH_2Cl_2 and centrifugation, resp. Aldonates and other ionic material were eliminated by passage through a column of Rexyn RG501 mixed bed ion exchange resin. The effluent consisting of the ion-free solution and a pyridine wash was evaporated to dryness.

Thin Layer Chromatography of Inositol Hexaacetate. The residue was acetylated under reflux for 1/2 hr with equal parts of pyridine and acetic anhydride. Following the addition of methanol to destroy excess acetic anhydride the solution was evaporated to dryness, extracted with CH_2Cl_2, and applied in a band to a glass plate coated with a layer of Kieselgur G, 250 microns thick. The chromatogram was developed twice in 2% MeOH/Hexane to the top of the plate to remove an artifact of the isolation procedure and twice in benzene to the middle of the plate where inositol hexaacetate

was visible as a band at the front. The band was scraped from the plate and heated under high vacuum at 175° for 1 hr in a cold finger sublimation apparatus. The crystalline sublimate was washed with a few drops of cold alcohol to remove yellow color and dissolved in 1.0 ml pyridine. [In a preliminary test of the isolation procedure the sublimate was identified after recrystallization from alcohol as inositol hexaacetate by melting point, 210°, unchanged when mixed with authentic compound, and by thin layer chromatography on silica gel in 4% MeOH/Benzene (Tate and Bishop, 1962)].

Quantitation of Inositol Hexaacetate. An aliquot of the pyridine solution containing about 50 μgm of compound was spotted on Kieselgur G and chromatographed as described above along with authentic inositol hexaacetate, 75 and 150 μgm. For measurement of acetyl groups the chromatogram was sprayed with $NH_2OH/Fe(NO_3)_3$ reagent (Tate and Bishop, 1962), modified by substitution of methanol for water. After thorough drying equal rectangular areas including each spot were scraped from the plate, suspended in 1.5 ml aqueous $Fe(NO_3)_3$ reagent, centrifuged, and read at 540 mμ against a blank prepared from another area of the chromatogram. Optical density was linear over the range of standards used.

Counting of Inositol Hexaacetate. The remainder of the pyridine solution was divided in half and each portion was banded on Kieselgur G and again chromatographed. Hexaacetate bands were removed and one was suspended in DPO/POPOP/toluene/Cab-O-Sil (Snyder and Stephens, 1962) and counted in the Packard Tricarb Liquid Scintillation spectrometer. The other was resublimed, chromatographed, assayed, and counted as described for the first portion. Within the error of the analytical method specific activity, expressed as counts per minute per mg inositol hexaacetate, was constant for the two stages of purification. [The increase in counting sensitivity realizable through the convenient handling of relatively large quantities of material is a distinct advantage of the thin layer chromatographic method. Between 2 and 3 mg of inositol hexaacetate containing

500 - 1,000 cpm were counted at each stage of purification.] Results of incubation of testis homogenate are seen in Table I, Experiments 1,2,3,4 and 6.

Regeneration of Inositol-C^{14}. Although aldoses are eliminated by oxidation and deionization, ketoses and hexitols are resistant to bromine oxidation. Exp. 5, Table I was designed to test the possibility that inositol hexaacetate was contaminated with acetates of labeled ketose and/or hexitol. Inositol hexaacetate isolated by thin layer chromatography and sublimation was dissolved with warming in 0.003 M methanolic CH_3ONa (Zemplén and Pacsu, 1929). Within an hour at room temperature crystalline inositol appeared. After standing overnight the crystals were washed free of reagent with $CHCl_3$ and dried.

Paper Chromatography of Inositol-C^{14}. A 0.2 mg aliquot of inositol-C^{14} was chromatographed descending on Whatman #1 paper in acetone/water, 85/15 (Posternak et al, 1955) for 9 hr. A scan of the inositol strip showed a single radioactive peak coterminous with a single $AgNO_3$/NaOH reactive spot (Trevelyan et al, 1950). Glucose, mannose, galactose, fructose, sorbitol, and authentic inositol were chromatographed on the same sheet. Inositol, $R_{glucose}$ 0.54, was clearly separated from the other compounds, $R_{glucose}$ 1.00, 1.11, 0.95, 1.10 and 0.98, resp. The radioactive product of these experiments is inositol-C^{14}.

Reacetylation of Inositol-C^{14}. The remainder of the crystalline inositol-C^{14} was acetylated, sublimed, and chromatographed on Kieselgur G. Counting of the inositol hexaacetate band showed no loss of radioactivity on reacetylation. (Exp. 5, Table I).

Rate of Synthesis. Fig. 1 shows the rate of inositol biosynthesis from glucose-U-C^{14} plotted as change of specific activity with time. Glucose-U-C^{14} and carrier inositol were incubated with testis homogenate in 5 flasks; at intervals of 0, 1/4, 1/2, 1, and 2 hr the reaction was stopped by heating and inositol, isolated as the hexaacetate, was counted at two stages of purification as described above. From the graph it is

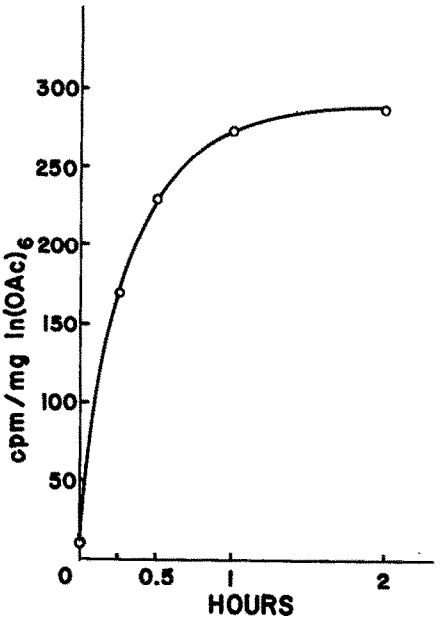

Fig. 1. The Rate of Biosynthesis of Inositol by Rat Testis Homogenate

seen that inositol reaches maximum specific activity in 1 hr. The specific activity at 2 hr represents an incorporation of 0.69%, Exp. 4, Table I.

Boiled Enzyme Control. In a companion to Experiment 6, Table I, a control experiment was performed in which an aliquot of KCl homogenate was heated for 15 min at 100° and then incubated with glucose-U-C^{14} and inositol. Isolated hexaacetate was devoid of radioactivity showing that inositol biosynthesis is mediated by a thermolabile factor.

Discussion. Although the use of glucose-U-C^{14} gives no insight into the mechanism of conversion of glucose to inositol, singly labeled glucose in the intact rat gave the same radiochemical yield of inositol regardless of location of the label (Imai, 1963), suggesting intact conversion of glucose. In kidney slices (Hauser and Finelli, 1963), on the contrary, a similar study gave variable incorporation suggesting synthesis from smaller units. Results of chemical degradation of inositol produced in vegetable systems suggest synthesis from intact hexose in plants (Loewus and Kelly, 1962) and from smaller units in yeast (Charalampous, 1957).

Studies designed to elucidate the mechanism of this reaction in a purified mammalian system are in progress in this laboratory.

Conclusion. Inositol synthesis has been observed for the first time in a cell-free system prepared from rat testis.

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