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The Synthesis of mesoInositol in Germ-free Rats and Mice

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The precise role of dietary mesoinositol in mammalian nutrition remains controversial despite more than two decades of inquiry. Early reports linking inositol to the promotion of growth and prevention of alopecia in mice (e.g. Woolley, 1940, 1941) and rats (e.g. Pavcek & Baum, 1941; Cunha, Kirkwood, Phillips & Bohstedt, 1943) have not been uniformly reduplicated (e.g. Martin, 1941; Fenton, Cowgill, Stone & Justice, 1950; Ershoff & McWilliams, 1943; McCormick, Harris & Anderson, 1954). In addition, no characteristic syndrome of inositol deficiency has yet been described in man. However, the recent findings that added inositol is required for the propagation of human cells in tissue culture (Eagle, Oyama, Levy & Freeman, 1957), and unbound inositol is concentrated in most mammalian tissues (Dawson & Freinkel, 1961) prompted a re-examination of the endogenous biosynthesis of inositol.

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Excellent relevant data were available in this area before the inception of the present studies. Needham's (1924) observations of sustained inositoluria in rats maintained on low inositol diets had been re-enforced by the independent reports of Daughaday, Lerner & Hartnett (1955) and Halliday & Anderson (1955) that [¹⁴C]inositol could be recovered from rats given repeated injections of [¹⁴C]glucose. However, as was first suggested by Woolley (1942), such 'endogenous' inositol could have originated from alimentary tract micro-organisms which are known to profoundly affect the picture in other fields of mammalian metabolism, e.g. the [¹⁴C]urea catabolism of mammals is entirely due to associated micro-organisms (Kornberg, Davies & Wood, 1954). To minimize intestinal contributions, Daughaday *et al.* (1955) excised the entire gut immediately before analysis of the carcass for inositol. The possibility of microbial biosynthesis and absorption of labelled inositol from the intestine during the 3 days

preceding killing was not thereby excluded. Similarly, although Halliday & Anderson (1955) effected prolonged reduction of intestinal flora by feeding antibiotics and caecectomy, the potential for inositol synthesis by the residual intestinal yeasts was not eliminated. An alternative approach was employed by Luckey, Pleasants, Wagner, Gordon & Reyniers (1955), who maintained complete intestinal sterility in two germ-free rats during chronic balance studies. However, owing to 'the variation in inositol analyses of different lots of diet', they were unable to interpret their recovery of 74 and 40% more inositol than had been fed as definite evidence for net biosynthesis.

For the present experiments, the isotopic and germ-free techniques were combined. [^{14}C]-Glucose was acutely administered to germ-free mice and rats and recovery of [^{14}C]inositol from the carcass was contrasted with values obtained under identical conditions in normal animals.

EXPERIMENTAL

Animals. Five days before the studies with radioactive glucose on 26 October 1959, germ-free rats (born 21 September 1959), germ-free mice (born 13 September 1959) and normal rats and mice (born 10 September 1959) were sent by air-express from the Lobund Institute, Notre Dame, Ind., U.S.A., to the Charles River Laboratories, Dover, Mass., U.S.A. A germ-free isolator (Reyniers, 1959) containing food, water, cages, dissection tools and cotton swabs was employed for the transport and subsequent maintenance of the germ-free animals. At the end of the [^{14}C]glucose experiments, swabbings were obtained from the inside walls of the isolator and from the surfaces and freshly expressed faeces of the germ-free animals. Sterile cultures from these swabbings in thioglycollate broth and on blood-agar plates indicated that contamination had not occurred during transport and maintenance of the animals at the Charles River Laboratories. All animals were generously donated by Professor P. C. Trexler of the Lobund Institute; maintenance of isolators and assistance with germ-free technique were generously provided by Dr H. L. Foster and his associates of the Charles River Laboratories.

Injection solutions. Chromatographically pure [^{14}C]-glucose (Nuclear-Chicago Corp., Ill.) was combined with unlabelled glucose (National Bureau of Standards) to prepare an injection solution containing 9.8 mg. of glucose and $30\mu\text{C}$ of ^{14}C /ml. The material was sterilized by autoclaving for 20 min. with 16 lb./in.² pressure. An ampouled portion together with appropriate syringes and needles was introduced into the isolator by passage through the supply lock, by using cold sterilization with a peracetic acid spray.

To establish that the [^{14}C]glucose was not contaminated with radioactive inositol, any contamination was concentrated by growing the inositol-dependent yeast *Kloeckera brevis* in the presence of the [^{14}C]glucose. A sample (1 ml.) of the glucose injection solution was added to the growth medium of Campling & Nixon (1954). Carrier inositol (4.8 μg .) was added and the mixture inoculated with *K. brevis*. The yield of *K. brevis* cells obtained after 3 days'

growth at 25° was normal as judged by turbidity readings (4.9 μg .). The cells, which previous experiments had shown contain all the inositol in combined form (R. M. C. Dawson & N. Freinkel, unpublished work), were hydrolysed with 6N-HCl for 40 hr. at 105°. To the hydrolysate was added 50 μg . each of inositol and glucose as carriers and it was then dried *in vacuo*. The residue was dissolved in water and passed through a column of mixed-bed ion-exchange resin, Amberlite MB-1 (8 cm. long $\times$ 0.8 cm. diameter). The effluent and washings were evaporated to low volume and applied as spots to two paper-strip chromatograms 2.5 cm. wide. These were developed with the solvents and conditions described below, and, after drying, passed through an automatic micromil-window strip scanner (Nuclear-Chicago Actigraph). The strips were treated with acetone-AgNO₃ and ethanolic NaOH reagents to detect the inositol and glucose spots (Trevelyan, Procter & Harrison, 1950). All of the radioactivity was confined to the glucose area. Under the conditions of the test 0.001% of the radioactivity occurring as *meso*inositol would readily have been detected.

METHODS

Experimental work on germ-free and normal animals. All studies were performed with male paired germ-free and normal animals. Samples of the glucose injection solution were administered at hourly intervals intraperitoneally as follows: one germ-free and one control rat were each given three 1 ml. injections; one germ-free and one control rat each received six 0.5 ml. injections; and one germ-free and one control mouse were each treated with three 0.5 ml. injections. The germ-free animals were injected by using the rubber manipulating gloves in the isolator. All animals were allowed free access to food throughout the experimental period. The animals were killed by a blow on the head 1 hr. after the last injection of each pair. Immediately thereafter, the animals were homogenized with 11N-H₂SO₄. Mice were introduced directly into 50 ml. of 11N-H₂SO₄ contained in a Waring Blendor. Separate blenders were employed for each animal. Rats were preliminarily sectioned into smaller pieces and ground with a meat grinder into 100 ml. of 11N-H₂SO₄/100 g. body wt. contained in Waring Blendors. Subsequent processing was by a modification of the method of Halliday & Anderson (1955).

The suspensions (animals homogenized with 11N-H₂SO₄) were hydrolysed in sealed bottles at 16 lb./in.² pressure for 12.3 hr. at 122°. The black hydrolysates were cooled and to each was added 25 mg. of carrier inositol. The hydrolysates were diluted with an equal volume of water and solid CaCO₃ was added in small portions until they were at about pH 5. The precipitate of CaSO₄ and other insoluble matter was filtered off under pressure and the precipitate washed well with water. The filtrate and washings were combined and decolorized with Darco G60 charcoal. This was most effectively carried out in the cold with successive small portions of charcoal, and filtering before using the next portion. Samples of the clear filtrate were retained for inositol assay (Campling & Nixon, 1954) as described by Freinkel, Dawson, Ingbar & White (1959). The remainder of the filtrates were passed through columns of mixed-bed ion-exchange resin, Amberlite MB-1 (2 in. diameter, 12 in. long for mouse experiments, 14 in. long for germ-free rat filtrates, and 17 in. long for control rats). The effluents and

washings were free of SO_4^{2-} ions when tested with Ba^{2+} ions. They were evaporated to low volume (about 0.5 ml.) and 4 ml. of ethanol was added to induce crystallization. The separated crystals were dissolved in 2 ml. of water. The mixture was centrifuged, and the supernatant separated from debris, evaporated to low volume (0.25–0.5 ml.) and treated with 2–3 ml. of ethanol. After the inositol had been allowed to crystallize for 1 hr. at 0° , it was collected and recrystallized from aqueous ethanol. The crystals were dried (e.g. for animal no. 6: melting point, 222.5° uncorr.). The samples of crystalline inositol were dissolved in 5 ml. of water. Samples (1 ml.) were evaporated on planchets for radioactive assay with an automatic micromil-window flow counter (Nuclear-Chicago Corp.) and a further sample (0.1 ml.) was used for microbiological inositol assay (Campling & Nixon, 1954).

Chromatography of inositol samples. Samples of the above inositol solutions equivalent to 1.2 mg. were run on paper chromatogram strips 5 in. wide. Two solvent systems were used: (1) propanol-ethyl acetate-water (24:13:7, by vol.) and (2) propan-2-ol-acetic acid-water (3:1:1, by vol.), both of which give a good separation of inositol from sugars such as glucose. After the chromatograms had been run (40 hr., descending) they were dried and a central strip $\frac{1}{4}$ in. wide was cut out and treated with the acetone- AgNO_3 and ethanolic NaOH reagents for detecting polyols (Trevelyan *et al.* 1950). Only an inositol spot was rendered visible, and the strips on either side of this were cut out of the untreated paper and extracted twice over 10 hr., each time with 20 ml. of water. A sample (0.05 ml.) was taken for inositol assay and the remainder (0.95 ml.) transferred to a planchet for counting.

For radioactive assay, sufficient counts were observed to reduce the probable error of the measurement to less than 3%. All planchets were appropriately corrected for self-absorption to a mass of 5 mg.

RESULTS

Results are summarized in Table 1. In the rats, total recovered inositol, after correction for added inositol carrier, averaged 27.7 mg./100 g. body wt. in the two germ-free, and 30.1 mg./100 g. body wt.

in the two normal, animals. The findings are in substantial agreement with the average values for carcass inositol of 28.1 mg./100 g. which Halliday & Anderson (1955) obtained in three normal rats by a similar fractionation procedure, and slightly greater than the 17.3 mg./100 g. (Daughaday *et al.* 1955) and 23.3 mg./100 g. (McCormick *et al.* 1954) recovered by somewhat different techniques. Incomplete recovery of inositol precluded estimation of total carcass inositol in the germ-free mouse. The value of 27.8 mg./100 g. in the control mouse is somewhat less than the average of 39.2 mg./100 g., which Woolley (1942) observed in six mice fed on a stock diet for 4 weeks after weaning.

Significant quantities of radioactivity were present in the inositol crystallized from the carcasses of all mice and rats after the administration of $[1-^{14}\text{C}]\text{glucose}$. That this constituted newly synthesized $[^{14}\text{C}]\text{inositol}$ was established for each animal by the demonstration of constant inositol specific radioactivity during chromatographic resolution in two solvent systems. In this determination of specific radioactivity the microbiological assay of inositol gave values within about $\pm 6\%$ and the radioactivity was determined within $\pm 3\%$. Further, the $[^{14}\text{C}]\text{inositol}$ isolated from one animal was used to support the growth of *K. brevis*. The yeast was then recovered from the culture medium, it was hydrolysed with acid and inositol was then isolated from the hydrolysate by strip chromatography. The specific radioactivity of the isolated and original inositol were identical (315 and 314 counts/min./mg. of inositol), confirming that the ^{14}C had been incorporated into *meso*inositol. Similar experiments reported in the Experimental section had already shown that the $[^{14}\text{C}]\text{glucose}$ injected was free of *meso* $[^{14}\text{C}]\text{inositol}$.

In the paired rats and mice killed 3 hr. after the first injection of $[1-^{14}\text{C}]\text{glucose}$, the total counts

Table 1. *The formation of radioactive inositol from $[1-^{14}\text{C}]\text{glucose}$ in vivo*

Animal	Weight (g.)	Duration of experiment (hr.)*	Recovered inositol (mg.)†	Recovery of [¹⁴ C]inositol				
				Counts/mg. of inositol/min.‡			Total counts	Per cent of [1- ¹⁴ C]glucose administered
				A	B	Average		
Rats								
Germ-free	104	3	51.5	138	126	132	6 798	0.0279
Control	180	3	75.5	131	134	132.5	10 004	0.0410
Germ-free	108	6	57.3	315	355	335	19 195	0.0787
Control	180	6	83.0	172	156	164	13 612	0.0558
Mice								
Germ-free	26	3	22.5	259	259	259	5 827	0.0478
Control	36	3	35.0	232	259	245.5	8 592	0.0704

* Interval elapsing between the first of a series of injections of $[1-^{14}\text{C}]\text{glucose}$ and killing. For details of administration schedules, see text.

† Before fractionation 25.0 mg. of carrier inositol was added to each carcass.

‡ Specific radioactivities of inositol (counts/min./mg.) isolated after paper chromatography of crystalline material in: (A) propanol-ethyl acetate-water, and (B) propan-2-ol-acetic acid-water. Average chromatographic specific activities were employed to derive minimal estimates of 'total counts' recovered as radioactive inositol from each animal.

recovered as radioactive inositol were greater in the control than in the germ-free animals. In the paired rats killed at 6 hr., total recovered radioactive inositol was greater in the germ-free animal. Paired values did not differ by more than 50 % in any instance, thus suggesting that intestinal microflora do not appreciably influence the fractional rates of endogenous inositol biosynthesis from administered [^{14}C]glucose in these species.

DISCUSSION

In the present experiments it has been established that the [^{14}C]glucose injected contains no contaminating *meso*[^{14}C]inositol. On the other hand, the crystalline *meso*inositol of the correct melting point isolated from the carcasses of both germ-free and normal animals after [^{14}C]glucose injection contained appreciable radioactivity. That this did not represent contamination of the isolated inositol with another metabolic product formed from the [^{14}C]glucose relies on the findings: (a) that the specific activity of the [^{14}C]inositol remained constant on paper chromatography in two solvent systems; (b) that it remained constant after a growth cycle through *K. brevis* and reisolation of the inositol from the yeast.

These results leave little doubt that normal and germ-free animals can biosynthesize labelled inositol, after the injection of [$1\text{-}^{14}\text{C}$]glucose. Since the completion of these studies, Eagle, Agranoff & Snell (1960) have reported the occurrence of definite, albeit limited, synthesis of inositol from radioactive glucose in isolated human tissue culture. The combined observations would indicate that mammalian tissues are capable of inositol production even without microbial synergy, and that all of the mammalian requirements for inositol need not be met exogenously. It cannot, however, be assumed without qualification that the unknown metabolic processes which synthesize [^{14}C]inositol result in a net formation of inositol which is available for the animal economy, for it is conceivable that if such processes are reversible, catabolism of inositol will outstrip synthesis.

Kinetics of glucose metabolism have been sufficiently documented in the rat (Feller, Strisower & Chaikoff, 1950) to justify efforts at more quantitative treatment of the present results. In control rats, 0.041 and 0.056 % of the counts in the administered [$1\text{-}^{14}\text{C}$]glucose could be recovered as radioactive inositol after experimental periods of 3 and 6 hr. respectively. The figures are in the same neighbourhood as the values of 0.05 and 0.07 % obtained by Halliday & Anderson (1955) when normal rats were killed 2 hr. after the completion of a series of ten injections of [$1\text{-}^{14}\text{C}$]glucose at 1 hr. intervals. It seems reasonable to assume

therefore that 0.05 % represents an approximate minimal figure for the conversion at equilibrium. Since the half-time for the turnover of glucose in the rat is less than 1 hr. (Feller *et al.* 1950), and since not enough glucose was injected at any time to disrupt equilibrium conditions, it might be anticipated that most of the labelled glucose in all of the studies had equilibrated with the exchangeable glucose pool. In the rat, turnover of body glucose amounts to 100 mg./hr./100 g. body wt. (Feller *et al.* 1950). Thus, taking 0.05 % as a minimal figure for the conversion at equilibration, this may be extrapolated for 24 hr. to estimate that a minimum of about 1.2 mg. of glucose/100 g. of rat is converted into inositol per day. Moreover, unless the metabolism and distribution of glucose are altered by intestinal sterility, the fact that fractional conversions of [$1\text{-}^{14}\text{C}$]glucose were of similar magnitude in the germ-free rats (i.e. 0.03 and 0.08 % at 3 and 6 hr. respectively) would suggest that the minimal estimate of 1.2 mg. may equally obtain in these animals. Unfortunately, translation of the values for glucose into actual quantities of inositol is precluded by the lack of information about pathways of inositol biosynthesis. If conversion of glucose into inositol were equimolar, then the results would be compatible with a minimum synthesis from glucose of 1.2 mg. of inositol/100 g. of rat/day. However, at least in yeast, such an equimolar transformation does not obtain (Charalampous, 1957), and consequently in this species the cyclization theory of inositol formation from glucose (Fischer, 1945) does not appear to be tenable. Thus, although the present studies constitute clear evidence of endogenous inositol biosynthesis, they do not resolve the question of the nutritional adequacy of this process.

SUMMARY

1. The biosynthesis of inositol from glucose has been examined in normal and germ-free rats and mice.
2. After the acute intraperitoneal administration of [$1\text{-}^{14}\text{C}$]glucose, [^{14}C]inositol could be isolated from all animals. In paired germ-free and control rats and mice, the recoveries of radioactive inositol were of the same order of magnitude.
3. The findings would indicate that inositol can be formed from glucose in these species and that endogenous biosynthesis does not require the presence of alimentary canal micro-organisms.

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Changes in Microsomal Components Accompanying Cell Differentiation of Pea-Seedling Roots

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This paper describes some changes found in the microsomes of pea roots during growth (see Whaley, Mollenhauer & Leech, 1960, for a review on plant microsomes). The growing-plant root provides a convenient source of material, because growth is largely in one direction and cell division is confined to the apex, so that it is possible to obtain batches of cells in progressive stages of development by cutting serial transverse segments. Small segments of the root tips were used, so that differences between the meristem and the first stages of differentiation could be studied. The experiments depend on the chance observation that the sedimented microsomal pellet may be readily separated into two components, one particulate and the other largely membranous. These components were examined by their protein and nucleic acid contents, by electrophoresis and by electron microscopy.

A preliminary communication of these results has been published (Loening, 1960).

MATERIALS AND METHODS

Preparation of root-tip segments. Pea seeds of the variety Meteor (Sutton and Sons, Reading) were sown in horticultural vermiculite-soft tap water (3:1, v/v) and ger-

minated in the dark at 25° for 48 hr. The roots were then 3–4 cm. long; exceptionally long or short ones were rejected. The roots were cut into three serial segments, the first tip segment being 1.6 mm. long, the second 1.8 mm. and the third basal segment 3.0 mm. To facilitate the cutting of large numbers of tips, a block of four Perspex sheets of thicknesses 1.6, 1.8, 3.0 and 6.0 mm. was drilled with 55 holes of suitable diameter to hold the roots. The block was placed, thinnest sheet downwards, flat on a sheet of glass and the roots were inserted into the holes so that the tips touched the glass. The protruding older parts of the roots were sliced off flush with the Perspex with a microtome blade and the block was inverted. The Perspex sheets were then removed one by one and the exposed segments sliced off. The whole operation took 10–15 min.

The lengths of the segments are chosen so that the first includes the meristem (potentially dividing cells) and the root cap, but few developed or elongating cells. The segment consists therefore of largely undifferentiated tissue. The second segment is a zone of increased metabolic activity, including protein synthesis (see Heyes & Brown, 1956). There is some cell enlargement and cell vacuoles appear. This segment therefore shows the beginnings of differentiation. The oldest part of the second segment and the youngest of the third is the zone with the most rapid rate of cell elongation. Cells of the third segment are very much larger and vacuolated, although the final cell volume is not reached until some 10–11 mm. from the tip. The average weight and number of cells per segment is shown in Table 1.