Studies on the Metabolic Role of *myo*-Inositol

DISTRIBUTION OF RADIOACTIVE *MYO*-INOSITOL IN THE MALE RAT

By LAWRENCE M. LEWIN, YSCA YANNAI, SOREL SULIMOVICI
and PERETZ F. KRAICER

*Department of Chemical Pathology and Department of Zoology, Tel Aviv University,
and Institute of Endocrinology, The Chaim Sheba Medical Center, Tel Hashomer, Israel*

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Radioactive *myo*-inositol was injected intraperitoneally into nephrectomized rats. The radioactive material present in liver, spleen, brain, heart, diaphragm, seminal vesicle, coagulating gland, prostate, epididymis, vas deferens and testis was shown to consist exclusively of *myo*-inositol and its derivatives, as shown by paper chromatography of hydrolysates and trichloroacetic acid extracts of these tissues. Radioactive *myo*-inositol was accumulated rapidly within 1 h by the thyroid, coagulating gland and seminal vesicle. Other tissues, such as the pituitary, prostate gland, liver and spleen, concentrated *myo*-inositol less actively. The muscle tissues studied (diaphragm and heart) concentrated little inositol, whereas brain, testis, and epididymal fat-pad did not concentrate it at all. The lipid fraction of liver contained most of the radio-labelled *myo*-inositol. In the other organs most of the radioactivity was found in the aqueous trichloroacetic acid extract, largely as free *myo*-inositol.

myo-Inositol is an essential growth factor for certain micro-organisms and for various animals, including the rat (Diem, 1962), as well as for human cells in tissue culture (Eagle *et al.*, 1957). Although evidence obtained from studies on a wide variety of organisms has indicated that *myo*-inositol was involved in cell morphogenesis and cytogenesis (Schopfer *et al.*, 1962), lipid synthesis (Challinor *et al.*, 1964; Lewin, 1965; Shafai & Lewin, 1968), cellular viability (Ridgway & Douglas, 1958; Lewin, 1965; Matile, 1966) and in the structure of cell membranes (Lewin, 1965; Matile, 1966), the role of this compound, at the molecular level, has not been elucidated.

myo-Inositol has been reported to be actively synthesized in seminiferous tubules of the rat (Eisenberg, 1967) and in the testicular spermatozoa of the ram (Voglmayr & White, 1971). Surgical cryptorchidism or the administration of triethylenemelamine to rats was accompanied by a decrease in *myo*-inositol biosynthesis, which paralleled the disappearance of spermatids and spermatozoa (Morris & Collins, 1971). This indicated that *myo*-inositol may play a role in spermatogenesis. In various species high concentrations of *myo*-inositol have been found in the rat testis, epididymal, vesicular and prostatic fluids (Mann, 1964; Voglmayr & Amann, 1973; Lewin & Beer, 1973), indicating that *myo*-inositol may play an important role as a constituent of the seminal fluid as well as in spermatogenesis.

The present work investigated the fate of radio-

active *myo*-inositol after intraperitoneal injection into mature male rats. In certain of these experiments bilaterally nephrectomized rats were used, because they have previously been reported to lack the capability to catabolize *myo*-inositol (Howard & Anderson, 1967). We have shown that all of the radioactivity in the nephrectomized animals was contained in the intact ring. It was possible, therefore, to study the uptake and distribution of this compound in the various organs, its subcellular distribution, its secretion in glandular fluids and other aspects of its biochemistry.

Materials

[2-¹⁴C]*myo*-Inositol (specific radioactivity 9.8 mCi/mmol) was from Calatomic Corporation, CA, U.S.A., and [2-³H]*myo*-inositol (specific radioactivity 4.5 Ci/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K. Radiochemical purities were verified by ascending chromatography by using reference samples on Whatman no. 1 paper and the solvent systems (a) and (c) as described below.

Solvent 100, PPO (2,5-diphenyloxazole) and dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] were from Packard Instrument Co., Inc., Downes Grove, IL, U.S.A. *myo*-Inositol was from BDH Chemicals Ltd., Poole, Dorset, U.K., and *myo*-inositol monophosphate was from Calbiochem, CA, U.S.A.

Methods

Radioactivity determinations

Radioactivity was measured in Packard Tri-Carb liquid-scintillation spectrometer (model no. 3310). The radioactivity of non-aqueous samples was determined in scintillation fluid consisting of 5g of PPO and 50mg of dimethyl-POPOP/litre of toluene. The radioactivity of aqueous samples was determined in scintillation fluid composed of 100g of naphthalene, 7g of PPO, 0.5g of dimethyl-POPOP, 20ml of ethylene glycol and 100ml of 2-ethoxyethanol made up to a final volume of 1 litre with dioxan. Counting efficiencies were determined by external standardization.

Treatment of experimental animals

Mature male albino rats (250–350g) of the Wistar strain were from Tel Aviv University, Ramat Aviv, Israel. The animals were maintained on Purina Lab chow *ad lib*.

When required, bilateral nephrectomy was performed, under diethyl ether anaesthesia, through an incision in the mid-line of the back, in such a manner as to leave the adrenal glands *in situ*. The incisions through the musculature and skin were closed with surgical thread and wound clips respectively. Similar incisions were made in sham-operated control rats, in which the kidneys were manipulated, but not excised.

myo-Inositol (1 or 10 μ Ci) dissolved in 2.0ml of sterile 0.85% NaCl was injected intraperitoneally into the rats. After various time-intervals, from $\frac{1}{2}$ to 24h after injection, the animals were killed by cervical dislocation, and the organs were removed and weighed. Portions of tissues were placed in glass vials, to which 2ml of Soluene 100 were added. The tissues were allowed to digest overnight at room temperature, and 13ml of the toluene-based scintillation fluid was added. The radioactivity was determined as described above.

Extraction of water-soluble and lipid fractions from organs

Portions (approx. 200mg wet wt.) of the organs were homogenized in 0.8ml of 6% (w/v) trichloroacetic acid, and the precipitate was separated from the supernatant fluid by centrifugation. The precipitate was re-extracted in the same manner. Samples (0.1ml) of the trichloroacetic acid extracts were placed in vials containing 2ml of Soluene 100, 13ml of the toluene-based scintillation fluid was added and the radioactivity was measured as described above.

The precipitate, after trichloroacetic acid extraction, was subjected to lipid extraction, by using the method of Folch *et al.* (1957) modified as follows. The precipitate was extracted with 3.8ml of chloroform/methanol (2:1, v/v). KCl (0.154M; 0.3ml) was added,

and the phases were separated by centrifugation. Portions of the lipid phase were evaporated to dryness in vials and dissolved in toluene-based scintillation fluid before measurement of their radioactivity.

Hydrolysis of organ fractions

Portions (approx. 50mg wet wt.) of the rat organs were placed in glass ampoules to which 0.4ml of 5M-HCl was added. The ampoule was then sealed and heated at 120°C for 16h (McKibbin, 1959). After cooling, the ampoule was opened and portions of the hydrolysates were used for paper chromatography. Gas chromatography was used to determine the total *myo*-inositol content of the organ by a method described by Lewin *et al.* (1973).

Paper-chromatographic methods

Portions of radioactive tissue hydrolysates or trichloroacetic acid extracts were applied to Whatman no. 1 chromatography grade filter paper and were developed at room temperature by the ascending procedure in the solvent systems (a) and (b), and by the descending procedure (for 4d) in solvent system (c). The chromatographic systems used were: (a) propan-2-ol/pyridine/0.1M-BaCl₂/ethyl acetate (8:8:4:1, by vol.); (b) propan-1-ol/methyl benzoate/acetic acid/water (7:3:2:5, by vol.); (c) aq. 88% (w/v) phenol; (d) 2,6-lutidine/propan-2-ol/water (3:2:2, by vol.).

Developed chromatograms were dried, the guide strips containing standard *myo*-inositol, scyllitol, glucuronic acid and its lactone, or *myo*-inositol 2-phosphate were treated by an alkaline AgNO₃ method for polyols (Trevelyan *et al.*, 1950) or by an ammonium molybdate dip for staining organic phosphate (Block *et al.*, 1955). The radioactive strip was cut into 20 pieces parallel to the origin. These were placed in plastic vials and the paper was moistened with 0.2ml of water. Dioxan-based scintillation fluid (10ml) was added and the radioactivity was measured. Vials with previously determined low background count rates were used.

Results

In preliminary experiments [¹⁴C]*myo*-inositol was injected into intact rats and the distribution of radioactivity among the various organs was compared, as described in the Methods section. It was found that certain organs (notably thyroid, coagulating gland and seminal vesicle) concentrated radioactive material in large amounts when compared with the blood on an equal net-weight basis. By contrast, other organs did not concentrate it at all. In order to establish, unequivocally, that what was being incorporated was the intact inositol ring system, it was desirable to work under conditions in which *myo*-inositol catabolism did not occur. Since it had been reported that the sole

Table 1. *Distribution of radioactivity in some organs of the male rat after intraperitoneal injection of [2-¹⁴C]myo-inositol*

Control (sham-operated) and nephrectomized animals were injected intraperitoneally with [2-¹⁴C]myo-inositol (1.0 μ Ci, specific activity 9.8 mCi/mmol) and were rapidly placed in a hermetically sealed metabolic cage. A stream of air was drawn through the cage before bubbling through flasks containing 70 ml of the CO₂-trapping reagent described by Jeffay & Alvarez (1961). Portions (0.5 ml) of the trapping reagent were mixed with Soluene 100 (1.5 ml) and toluene-based scintillation fluid and the radioactivity was measured as described in the Methods section. The rats were killed 5 h after injection of inositol. Portions of the organs listed below were digested with Soluene 100 and the radioactivity was measured, as described in the Methods section. The results show the averages ($\pm$ S.D.) obtained for each group of four rats.

Organ	Distribution of radioactivity			
	Nephrectomized rats		Sham-operated rats	
	(d.p.m./mg wet wt.)	(% of administered dose)	(d.p.m./mg wet wt.)	(% of administered dose)
Blood serum	3.1 $\pm$ 0.2	—	2.3 $\pm$ 0.6	—
Brain	2.2 $\pm$ 0.5	0.16 $\pm$ 0.03	6.2 $\pm$ 1.5	0.42 $\pm$ 0.09
Heart	12.3 $\pm$ 1.8	0.39 $\pm$ 0.07	9.3 $\pm$ 1.9	0.28 $\pm$ 0.07
Diaphragm	5.9 $\pm$ 0.7	0.16 $\pm$ 0.03	6.7 $\pm$ 2.4	0.16 $\pm$ 0.09
Liver	38.7 $\pm$ 4.8	14.90 $\pm$ 2.06	24.1 $\pm$ 5.2	10.05 $\pm$ 2.3
Spleen	29.5 $\pm$ 5.8	0.65 $\pm$ 0.05	19.7 $\pm$ 3.8	0.7 $\pm$ 0.06
Pituitary	73.6 $\pm$ 31.8	0.03 $\pm$ 0.01	42.1 $\pm$ 5.3	0.01 $\pm$ 0.005
Thyroid	286.0 $\pm$ 80.0	0.08 $\pm$ 0.02	109.8 $\pm$ 38.4	0.05 $\pm$ 0.01
Kidney	—	—	94.5 $\pm$ 10.4	8.01 $\pm$ 0.43
Testis	3.7 $\pm$ 0.5	0.47 $\pm$ 0.07	3.6 $\pm$ 0.7	0.42 $\pm$ 0.05
Vas deferens	36.3 $\pm$ 5.0	0.26 $\pm$ 0.02	23.6 $\pm$ 3.7	0.18 $\pm$ 0.02
Epididymis	41.9 $\pm$ 3.1	1.66 $\pm$ 0.23	17.6 $\pm$ 3.7	0.7 $\pm$ 0.15
Coagulating gland	143.6 $\pm$ 34.7	—	61.8 $\pm$ 17.1	0.25 $\pm$ 0.09
Seminal vesicle	134.9 $\pm$ 23.1	2.04 $\pm$ 0.48	64.5 $\pm$ 14.6	0.98 $\pm$ 0.2
Prostate	51.7 $\pm$ 14.9	0.65 $\pm$ 0.38	29.8 $\pm$ 7.1	0.33 $\pm$ 0.7
Epididymal fat-pad	4.0 $\pm$ 1.1	—	2.4 $\pm$ 0.9	—
Urine	—	—	—	0.84 $\pm$ 0.48
Expired CO ₂	—	0.27 $\pm$ 0.09	—	15.76 $\pm$ 5.47

Table 2. *Paper chromatography of myo-inositol and some of its metabolites*

Chromatography was performed as described in the Methods section.

Solvent system ... Compounds	<i>R_F</i> values		
	(a)	(b)	(c)
myo-Inositol	0.30	0.24	1.0*
scyllo-Inositol	—	—	0.65*
D-Glucuronic acid	0.15	0.34	—
D-Glucuronolactone	0.83	0.50	—
myo-Inositol 2-phosphate	0.0	0.15	0.20*

* *R_F* values: defined as the ratio of the distance from the origin of the centre of the spot to the distance travelled by known myo-inositol run under the same conditions.

production by the rat, after injection of [2-¹⁴C]myo-inositol, confirmed that the bilaterally nephrectomized rats were essentially unable to convert inositol into CO₂, whereas the sham-operated control rats catabolized about 16% of the injected inositol to CO₂ in 5 h. The control animals excreted approx. 0.84% of the administered radioactivity in the urine, and paper chromatography revealed that only part of this was in the form of myo-inositol. The catabolism of inositol by the kidney thus appeared to be quantitatively more significant than its excretion in urine. These results confirmed the similar findings of Howard & Anderson (1967). In our experiments nephrectomized rats were still alive and vigorous at least 48 h after the operation, but no animal in our series was used more than 24 h after nephrectomy.

Trichloroacetic acid extracts of some organs of nephrectomized rats (i.e. liver, spleen, heart, diaphragm, brain, testis, epididymis, vas deferens, prostate, coagulating gland and seminal vesicle) were prepared and subjected to paper chromatography in the solvent systems (a)–(c) described in the Methods section. These solvents separated inositol from its common metabolites (Table 2). In all of the organs, except for epididymis and liver, the only radioactive

pathway of inositol catabolism in the rat takes place in the kidney (Howard & Anderson, 1967), further experiments were conducted to determine the suitability of bilaterally nephrectomized rats as experimental animals in these investigations.

Experiments (Table 1) in which we measured ¹⁴CO₂

Table 3. Incorporation of [2-³H]myo-inositol into trichloroacetic acid extracts and lipid fractions of organs of nephrectomized rats

Portions (approx. 100mg wet wt.) of organs obtained 8, 16 or 24h after the injection of [2-³H]myo-inositol (10 μ Ci, specific activity 4.5 Ci/mmol) were extracted with 6% (w/v) trichloroacetic acid (a) and chloroform/methanol (2:1, v/v) (b), and the radioactivity in the extracts was measured, as described in the Methods section.

Organ	Time after injection (h) ...	10 ⁻³ × Radioactivity (d.p.m./g wet wt. of tissue)					
		8		16		24	
		(a)	(b)	(a)	(b)	(a)	(b)
Testis		43	1	—	—	21	4
Epididymis		66	7	—	—	—	—
Vas deferens		82	8	—	—	95	22
Prostate		147	16	345	28	302	13
Coagulating gland		632	9	—	—	1700	127
Seminal vesicle		237	8	325	18	531	56
Brain		29	1	—	—	35	3
Spleen		69	12	82	33	108	30
Liver		48	68	60	138	58	66
Diaphragm		34	1	—	—	19	7

Fig. 1. Uptake of [2-³H]myo-inositol by some organs of the nephrectomized male rat after intraperitoneal injection

Test animals were injected intraperitoneally with [2-³H]-myo-inositol (10 μ Ci, specific activity 4.5 Ci/mmol), and killed at the intervals shown. Portions of the organs were digested with Soluene 100 and the radioactivity measured, as described in the Methods section. Seminal vesicle (x); brain (o); liver (●); blood (+); heart (□).

zones on the chromatograms had the R_f value corresponding to that of myo-inositol. The trichloroacetic acid extracts of epididymis and liver contained a small percentage of material whose R_f value corresponded to that of inositol monophosphate, in addition to the major inositol peak. In further experiments we subjected samples of the organs of nephrectomized rats to conditions designed to hydrolyse inositol derivatives to free inositol. The resulting hydrolysate was chromatographed on paper in solvent system (a). Essentially all of the radioactivity in the hydrolysates, including those from the epididymis and the liver, was found in the chromatographic zone corresponding to standard myo-inositol. The distribution of radio-labelled inositol between trichloroacetic acid-

soluble fractions and lipid fractions was studied with various rat organs. Table 3 shows the results of typical experiments, in which the bulk of the radio-labelled inositol in most of the organs was found in trichloroacetic acid-soluble form, in contrast with the situation in the liver, where inositol lipid formed the major portion (approx. 60%) of the accumulated radio-labelled material.

In a series of experiments (Fig. 1) the radioactivity of selected organs of nephrectomized rats was measured over a period of 24h after injection of ³H-labelled myo-inositol. Inositol uptake was rapid, being essentially complete 1–2h after intraperitoneal injection. Table 1 shows the distribution of radioactivity among organs of nephrectomized and sham-operated rats 5h after injection of [¹⁴C]myo-inositol. The brain, which had a high total inositol concentration (696 μ g/g wet wt.) apparently was not capable of concentrating radioactive inositol from the blood. The adipose tissue (epididymal fat-pad) was also unable to do so. Muscle tissues (heart and diaphragm) accumulated small amounts of inositol. The liver, spleen, pituitary gland and especially the thyroid glands concentrated radioactive myo-inositol from the blood quite actively. Special attention was given to the organs of the male reproductive tract, since they are known to contain high myo-inositol concentrations (Eisenberg & Bolden, 1964). The testes did not concentrate radio-labelled inositol from the blood; the vas deferens and epididymis accumulated more than 12-fold the blood serum concentration, whereas the coagulating gland (46-fold), seminal vesicle (43-fold) and prostate (16-fold) were quite active in taking up radio-labelled inositol under the conditions of our experiments.

The distribution of radioactivity among the organs of sham-operated rats was different from that in the nephrectomized animals. The kidney concentrated radio-labelled *myo*-inositol, which confirms the findings of Howard & Anderson (1967) and of Hauser (1963). The sham-operated rats accumulated less radioactivity in most of the organs tested, presumably because significant amounts of the administered inositol were metabolized or excreted by the kidney. An interesting exception to the rule was the brain, which accumulated more radioactivity in sham-operated rats than in nephrectomized animals.

Discussion

The importance of the kidney in the metabolism of *myo*-inositol by the rat and man has been shown by the work of Charalampous & Lyras (1957), Howard & Anderson (1967), Hankes *et al.* (1969), Perles & Colas (1960), Clements *et al.* (1973) and Melmed *et al.* (1974). In this study the paper-chromatographic solvent systems that were used were designed to separate inositol from its known catabolic products (glucuronic acid and its further metabolites) (Puhukainen & Hanninen, 1973) as well as from *scyllo*-inositol and inositol monophosphate. The results showed that the radio-labelled compounds found in organs from nephrectomized rats contained the *myo*-inositol ring, and consequently measurement of radioactivity was used to determine the distribution of *myo*-inositol within the rat.

The low concentration of radioactive inositol in the brain of nephrectomized rats, as compared with the concentration in blood serum, indicated the presence of a limited rate of transfer for inositol between blood and brain in the rat. The higher amounts of radioactivity measured in the brains of control rats (Table 1) may be due to the presence of metabolites of inositol, produced by the kidney, which are better able to cross the blood/brain barrier than is inositol. The presence of a blood/brain barrier for inositol raises a puzzling question concerning the source of the high concentration of inositol found in cerebrospinal fluid (Baumgarten & Saar, 1960; Garcia-Bunuel & Garcia-Bunuel, 1968; Lewin *et al.*, 1973) and brain tissue, since the inositol biosynthesis pathway reported by Eisenberg (1967) was not very active in rat brain. Dawson & Freinkel (1961) noted a high concentration of unesterified inositol in rabbit brain. Spector & Lorenzo (1975) have reported that entry of inositol from the blood into the central nervous system of the rabbit is regulated by a saturable transport system and the locus of this system may be the choroid plexus. We have also observed an inability to concentrate inositol in the rat testis (Lewin & Sulimovici, 1975).

In marked contrast with the testis, the other organs of the male reproductive tract that we measured (the

epididymis, vas deferens, prostate gland, seminal vesicle and coagulating gland) were all active concentrators of *myo*-inositol. Since the volume of fluid from the seminal vesicle was much greater than that from the other glands, it would appear to be the major source of seminal fluid inositol in the rat. The results of studies of intracellular distribution of *myo*-inositol (Lewin & Sulimovici, 1975) showed that most of the inositol in the seminal vesicle and prostate was found in the high-speed supernatant fraction. This may reflect inositol in transit, which will later be secreted in the glandular fluid. Paper chromatography demonstrated that the radioactive material in the seminal vesicle fluid from nephrectomized rats consisted exclusively of inositol. By contrast with the subcellular distribution in the seminal vesicle and prostate, most of the inositol was present in particulate fractions of liver (Lewin & Sulimovici, 1975; Maibauer & Herken, 1956). The results reported here for trichloroacetic acid and lipid extracts (Table 3) are consistent with the subcellular distribution data. The radio-labelled inositol lipids, which are presumed to be membrane-bound, were major components of the liver radioactivity, but minor constituents in the other organs tested. It was noted that the liver accumulated approx. 15% of the total injected radioactive inositol (Table 1). Perhaps the incorporation of inositol into lipids in the liver can provide a useful mechanism to store inositol in the rat.

It has been suggested by various workers that the essential role of *myo*-inositol in cell physiology might be explained by the inositol phospholipids that are present in cellular and subcellular membranes (see review by Lewin, 1967). The high concentration of free inositol in some rat organs may indicate that this compound plays an important role other than as a component of inositol lipids, in the function of the cell.

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