Carbohydrate Research
Elsevier Publishing Company, Amsterdam
Printed in Belgium

BIOSYNTHESIS OF L-ASCORBIC ACID FROM *myo*-INOSITOL

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(Received April 13th, 1970; accepted for publication in revised form, June 19th, 1970)

ABSTRACT

L-Ascorbic acid biosynthesis from *myo*-inositol was studied in hen-kidney and rat-liver slices and homogenates. For comparison, D-glucuronic acid, D-glucurono-6,3-lactone, and L-gulono-1,4-lactone were used as substrates, in order to clarify the metabolic significance of the *myo*-inositol pathway in L-ascorbic acid biosynthesis.

A paper-chromatographic method was developed for the separation of labelled L-ascorbic acid from the substrate, *myo*-inositol, and other glucuronic acid pathway metabolites. The glucuronolactonase activity was determined by recording the pH changes due to the liberation of protons during lactone hydrolysis.

Hen-kidney slices were able to synthesize L-ascorbic acid from *myo*-inositol as from D-glucuronic acid, D-glucurono-6,3-lactone, and L-gulono-1,4-lactone. The rate of L-ascorbic acid synthesis from *myo*-inositol was so high that this pathway is biologically significant. Rat-liver slices and homogenates were unable to use *myo*-inositol as a substrate due to the lack of *myo*-inositol oxygenase, whereas other metabolites were converted into L-ascorbic acid.

INTRODUCTION

Most animals are able to synthesize L-ascorbic acid. The synthesis starts from D-glucose, which *via* D-glucuronic acid and L-gulono-1,4-lactone is converted into L-ascorbic acid. D-Glucuronic acid can be obtained from UDP-GA [uridine 5'-(D-glucosyluronic acid dihydrogen pyrophosphate)] by hydrolysis *via* D-glucuronic acid 1-phosphate by UDP-GA pyrophosphatase and glucuronic acid-1-phosphatase. It is also liberated by β-glucuronidase in the hydrolysis of β-D-glucopyranosiduronic acids and D-glucuronic acid heteroglycans, which are both synthesized from UPD-GA. The biological significance of these three routes for formation of D-glucuronic acid in L-ascorbic acid biosynthesis is still under discussion. The level of glucuronic acid-1-phosphatase is very low or the enzyme is totally absent in the liver, which carries out the L-ascorbic acid synthesis in mammals, and the pH optimum of β-glucuronidase is very low[1,2]. In addition to the UDP-GA pathway, D-glucuronic acid can also be synthesized from D-glucose *via myo*-inositol, with the aid of *myo*-inositol oxygenase[3]. Most of the D-glucuronic acid thus synthesized is, however, metabolized in mammals

to pentose[4]. L-Ascorbic acid synthesis takes place, *e.g.* in rat liver, where *myo*-inositol oxygenase activity is very low or totally absent[2,5], and *myo*-inositol is catabolized in the kidneys[6], which lack L-gulono-1,4-lactone oxidase[2].

L-Ascorbic acid is synthesised in hen kidney[7], and hen-kidney extracts were found to have *myo*-inositol oxygenase activity. A study was therefore undertaken to ascertain whether *myo*-inositol was converted into L-ascorbic acid by hen-kidney slices and extracts and to determine the flux and biological significance of this route. Rat liver was studied for comparison, although no, or minimal amounts of, labelled L-ascorbic acid has been detected in urine after the *in vivo* administration of labelled *myo*-inositol to rats[5].

The data obtained indicate that *myo*-inositol can be converted into L-ascorbic acid by hen kidneys but not by rat liver, although both tissues are able to synthesize L-ascorbic acid from D-glucuronic acid, D-glucurono-6,3-lactone, and L-gulono-1,4-lactone.

METHODS

Nine white Leghorn hens (about six months old) and ten male Wistar rats (about three months old) fed *ad libitum* were used. The animals were decapitated and bled. The kidneys of the hens and the liver of the rats were cooled in ice-cold, oxygen-saturated, Krebs–Ringer phosphate medium. After cooling, 0.25-mm thick slices were cut in a Mickle Laboratory Engineering microtome. Some of the tissues were also homogenized by an Ultra Turrax homogenizer in a volume of 0.15M potassium chloride four times the fresh weight of tissue.

The tissue slices, *ca.* 5 mg dry weight, were incubated in conical centrifuge tubes under oxygen atmosphere at 38° with continuous shaking[8]. The volume of the Krebs–Ringer phosphate medium was 0.3 ml, and it contained, in the experiments with labelled *myo*-inositol, 10 μmoles of unlabelled *myo*-inositol and 0.8 nmole of ^{14}C-labelled *myo*-inositol (312 Ci/mole; The Radiochemical Centre, Amersham, England). When unlabelled D-glucuronic acid, D-glucurono-6,3-lactone, and L-gulono-1,4-lactone were compared with *myo*-inositol as substrates in L-ascorbic acid biosynthesis, 5 μmoles of each compound was added to the reaction mixture. The incubation was stopped by adding 25 μl of conc. hydrochloric acid, followed by 2.5 μmoles of unlabelled, carrier L-ascorbic acid, when labelled L-ascorbic acid was studied. A glass rod was used to crush the slices. The reaction mixtures were centrifuged to separate the denatured tissue fragments. When unlabelled substrates were used, 1 ml of 1% (w/v) oxalic acid was added before spinning to increase the volume. L-Ascorbic acid was determined in the supernatant by 2,6-dichlorophenol–indophenol titration[2]. The sedimented, denatured tissue was dried overnight at 90° and weighed.

The respiration of hen-kidney slices in the presence and absence of *myo*-inositol (33 mM) was controlled as described earlier[8].

When tissue homogenates were used in the studies of L-ascorbic acid biosynthesis, the reaction mixture contained 1 ml of 0.2M potassium phosphate, 50mM potassium cyanide buffer (pH 6.5), 0.1 ml of 0.2M solutions of the different substrates,

and 0.1 ml of tissue homogenate. The reaction was carried out at 38°, and it was stopped by adding 1 ml of 4.5% (w/v) aqueous trichloroacetic acid. The denatured protein was separated by centrifuging, and L-ascorbic acid was determined by titration. Protein determinations were carried out by the biuret reaction[9].

The separation of the various metabolites of *myo*-inositol and L-ascorbic acid was achieved by descending chromatography on Whatman No. 1 paper with butyl alcohol–acetic acid–water (120:30:50) (Fig. 1); 100 μl of the supernatant fraction was applied to the paper followed by a 36-h run. The standard compounds were located with aniline–oxalic acid at 120° for 10 min[10]. L-Gulono-1,4-lactone and *myo*-inositol were located with alkaline silver nitrate[10]. The L-ascorbic acid spot was located with the help of the reference spots. The radioactivity was determined in a scintillation solution containing 3.2 g of 2,5-diphenyl-4-oxazolone and 80 mg of *p*-bis(*o*-methylstyryl)benzene per litre of toluene in a Decem-NTL-314-scintillation spectrometer (Wallac Co., Turku, Finland).

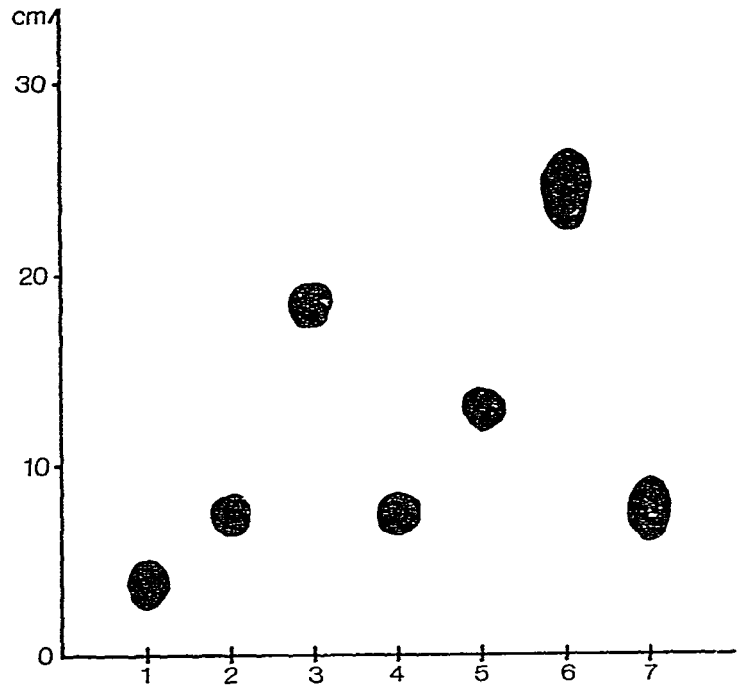

Fig. 1. The separation of L-ascorbic acid from *myo*-inositol and its metabolites by descending paper chromatography: 1 = *myo*-inositol, 2 = D-glucuronic acid, 3 = D-glucurono-6,3-lactone, 4 = L-gulonic acid, 5 = L-gulono-1,4-lactone, 6 = L-ascorbic acid, and 7 = L-*xylo*-hexulosonic acid, 10 μg of each run on Whatman No. 1 paper for 36 h in butyl alcohol–acetic acid–water (120:30:50). The scale is indicated by the Y-axis.

For the recrystallization, the L-ascorbic acid-containing spots were eluted with dilute hydrochloric acid, and 1 g of carrier L-ascorbic acid was added to the solution. The water was removed *in vacuo* at 40°, and the residue was dissolved in 5 ml of 90%

(v/v) aqueous ethanol. L-Ascorbic acid was recrystallized in the cold three times, and the specific activity was determined.

myo-Inositol oxygenase activity was measured from the tissue homogenates as described earlier[2].

For the determination of glucuronolactonase activity, tissue homogenate or its microsomal and soluble fractions[2] were added to a final volume of 4 ml of a reaction mixture containing 120 μmoles of D-glucurono-6,3-lactone and 10 μmoles of potassium phosphate buffer to bring the initial pH to 7.15. Before the enzyme preparation was added, the nonenzymic hydrolysis of the lactone was recorded. The reaction was followed for 15 min at room temperature by recording the pH decrease by a Radiometer model PHM 72 pH-meter connected to a Servogor RE 511-recorder calibrated to record 0.5 pH-units per 20 cm. By adding different amounts of free D-glucuronic acid to the reaction mixture under the same conditions, a calibration curve was obtained and found to be linear (Fig. 2A).

The content of free inositol in hen kidneys was determined from 5% (w/v) aqueous trichloroacetic acid extracts of the tissue. Other sugars were separated as described by Eagle and co-workers[11], and inositol was determined by the metaperiodate method[12].

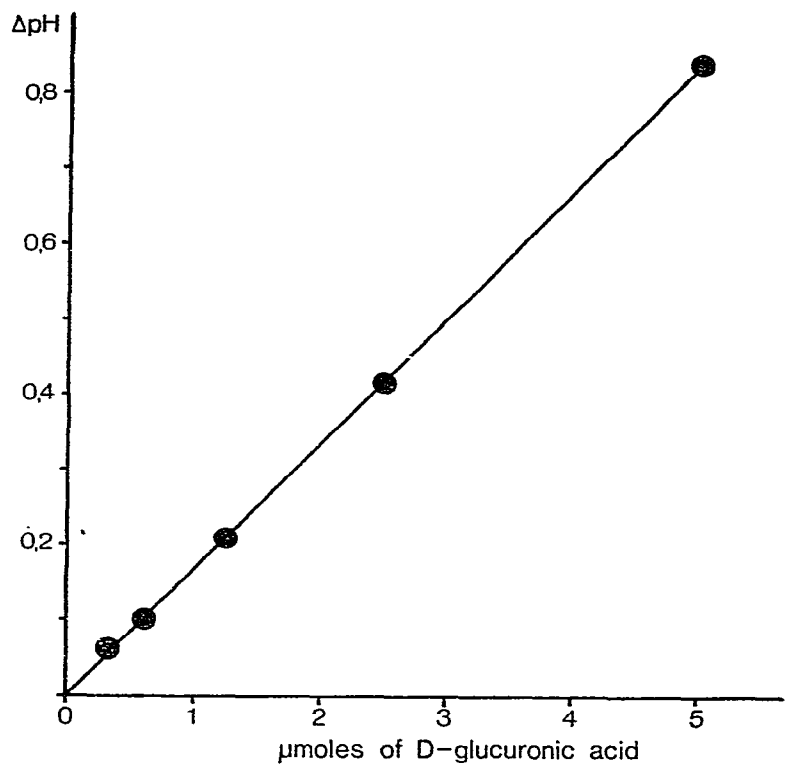

Fig. 2A.

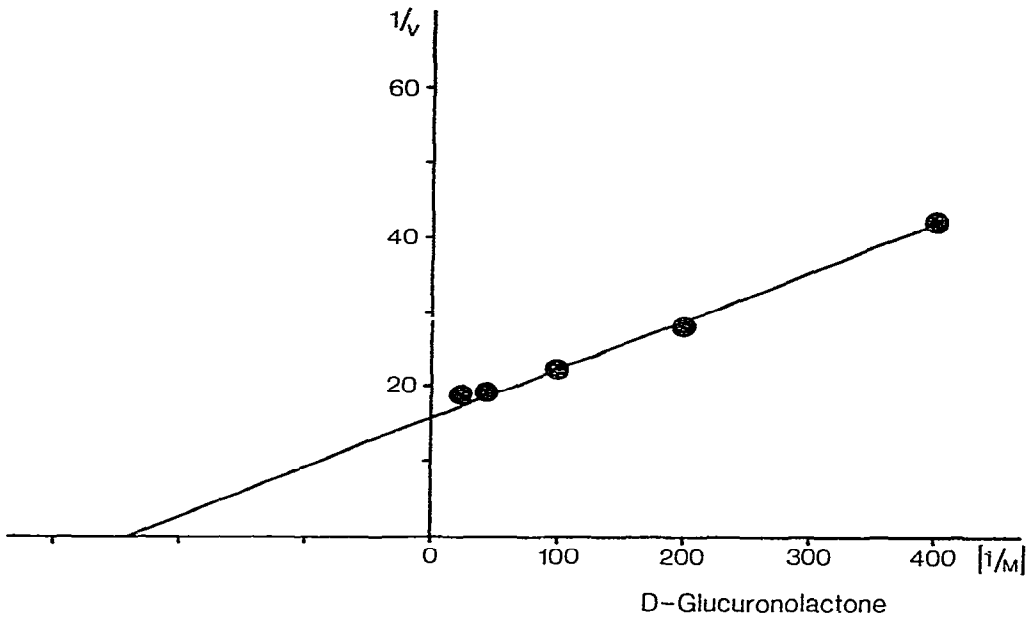

Fig. 2B.

Fig. 2. The determination of glucuronolactonase activity by recording (with the aid of a pH-meter) the release of protons during hydrolysis. A. D-Glucuronic acid standard curve obtained by adding various amounts of D-glucuronic acid to 4 ml (final volume) of 2.5mM potassium phosphate and uncentrifuged, hen-kidney homogenate (3.6 mg of protein). The initial pH was 7.150. B. The Lineweaver–Burk plot of glucuronolactonase of the hen-kidney soluble fraction (105,000 *g*, 60 min; 3.6 mg of protein per reaction mixture), giving a K_m value of 4.2×10^{-3}M.

RESULTS

Hen-kidney slices were able to convert *myo*-inositol, D-glucuronic acid, D-glucurono-6,3-lactone, and L-gulono-1,4-lactone to L-ascorbic acid. *myo*-Inositol was nearly as good a substrate as D-glucuronic acid and D-glucurono-6,3-lactone, whereas L-gulono-1,4-lactone was a much better substrate than *myo*-inositol. The results indicate that the *myo*-inositol oxygenase step is not completely rate-limiting neither is the uronolactonase step (Table I).

TABLE I

THE BIOSYNTHESIS OF L-ASCORBIC ACID BY HEN-KIDNEY AND RAT-LIVER SLICES FROM *myo*-INOSITOL AND ITS METABOLITES IN KREBS–RINGER PHOSPHATE MEDIUM

Substrate (*16.7*mM)	L-*Ascorbic acid synthesized* (*μmoles/h for each g of dry wt.*)	
	Hen-kidney slices	*Rat-liver slices*
myo-Inositol	4.1	none
D-Glucuronic acid	6.9	1.6
D-Glucurono-6,3-lactone	6.1	1.2
L-Gulono-1,4-lactone	53.2	3.3

Experiments with labelled *myo*-inositol showed that it was indeed converted into L-ascorbic acid by hen-kidney slices. The reaction proceeded linearly for at least one hour (Fig. 3). During this period, *ca.* 0.4% of the added *myo*-inositol was converted into L-ascorbic acid. Recrystallization of the L-ascorbic acid synthesized and separated from other metabolites gave 1768 after the first, and 1992 and 1980 counts/min/g of L-ascorbic acid after the second and third recrystallizations, respectively. These figures indicate that the paper-chromatographic separation had been effective.

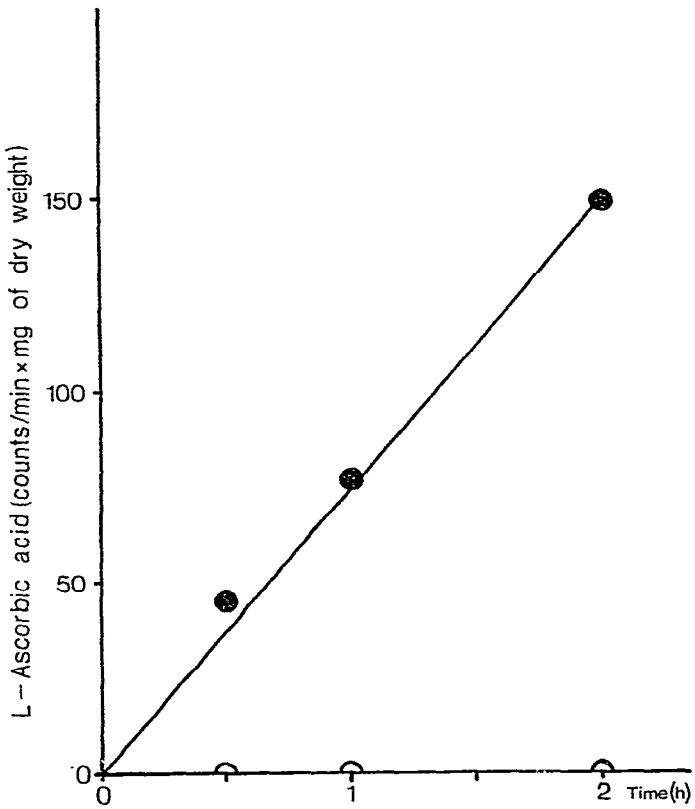

Fig. 3. The synthesis of labelled L-ascorbic acid from labelled *myo*-inositol in hen-kidney (—●—) and rat-liver (–O–) slices.

The addition of *myo*-inositol into the reaction mixture had no harmful effects on the respiration of the hen-kidney slices. The oxygen uptake was 0.52 and 0.53 mmole/h for each g of dry tissue in the presence and absence of *myo*-inositol (33 mM), respectively.

Hen-kidney extracts were able to catalyze L-ascorbic acid synthesis from D-glucuronic acid, D-glucurono-6,3-lactone, and L-gulono-1,4-lactone in the presence of phosphate–cyanide buffer. D-Glucurono-6,3-lactone was as good a substrate as L-gulono-1,4-lactone, although the tissue slices used the latter much more readily.

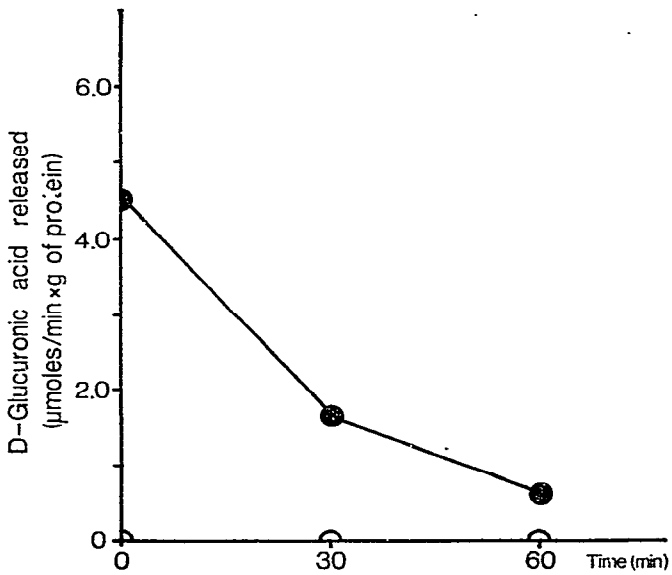

Fig. 4. The effect of incubation under an oxygen atmosphere at 38° on the *myo*-inositol oxygenase activity in the hen-kidney (–●–) and rat-liver (–○–) extracts fractionated with ammonium sulphate[2]. The first determination was carried out 30 min after the death of the animal.

myo-Inositol was not, however, a substrate in the presence or absence of cyanide (Table II).

TABLE II

THE CONVERSION OF *myo*-INOSITOL AND ITS METABOLITES INTO L-ASCORBIC ACID, CATALYZED BY HEN-KIDNEY AND RAT-LIVER HOMOGENATES IN THE PRESENCE AND ABSENCE OF CYANIDE

Substrate (*16.7*mM)	L-*Ascorbic acid synthesized* (*μmoles/h for each g of protein*)	
	Hen-kidney homogenates	*Rat-liver homogenates*
In the presence of 50mM *cyanide:*		
myo-Inositol	none	none
D-Glucuronic acid	2.2	1.2
D-Glucurono-6,3-lactone	50.2	34.4
L-Gulono-1,4-lactone	48.8	32.0
In the absence of cyanide:		
myo-Inositol	none	
D-Glucurono-6,3-lactone	0.84	

Rat-liver slices could convert D-glucuronic acid, D-glucurono-6,3-lactone, and L-gulono-1,4-lactone, but not *myo*-inositol, into L-ascorbic acid. The rate of L-ascorbic acid synthesis from the precursors in rat-liver slices was much slower than in hen-kidney slices (Table I). Experiments with labelled *myo*-inositol indicated that

no labelled L-ascorbic acid was synthesized from this substrate (Fig. 3). The same results were observed with rat-liver homogenates, which were otherwise almost as active as hen-kidney extracts in catalyzing L-ascorbic acid synthesis from D-glucuronic acid, D-glucurono-6,3-lactone, and L-gulono-1,4-lactone (Table II).

Hen-kidney extracts had *myo*-inositol oxygenase activity. The enzyme was rather labile, and it was rapidly inactivated when the preparation was incubated at 38° (Fig. 4). *myo*-Inositol oxygenase activity could not be demonstrated in the rat-liver extracts. Hen-kidney extracts appeared also to have an active glucuronolactonase in the soluble fraction (60 min, 105,000 *g*) of the extracts, but the microsomal fractions were inactive (Fig. 2B).

In order to study the *in vivo* relevance of the L-ascorbic acid synthesis by hen-kidney slices, the *myo*-inositol was determined from hen kidneys. A value of 3 μmoles/g of fresh weight was obtained.

DISCUSSION

The results indicate that hen kidneys are able to synthesize L-ascorbic acid *via* the *myo*-inositol–D-glucuronic acid pathway, and that *myo*-inositol is a biologically significant substrate in the L-ascorbic acid biosynthesis in hens. By using the data obtained from the experiments with labelled *myo*-inositol, a value of 1.9 μmoles/h for each g of dry weight is found for the flux in this pathway under the *in vitro* conditions used in this study. Since the fresh weight of hen's kidneys was 12–20 g, L-ascorbic acid could be synthesized by the kidneys *in vitro* at a rate up to10 μmoles/h (240 μmoles/day). The *myo*-inositol concentration in the hen-kidney tissue was found to be approximately 3 mM (3.3 and 6.9 μmoles/g of kidney in rats, 5.9 in guinea pigs, and 4.8 in rabbits[13,14]), which means that the *myo*-inositol pathway can probably satisfy a greater part of the daily L-ascorbic acid requirement of the hen.

The data obtained showed that *myo*-inositol oxygenase is only partly rate-limiting in the use of *myo*-inositol as substrate in L-ascorbic acid biosynthesis in hen-kidney slices, since the rate of synthesis from the reaction product D-glucuronic acid was only 1.7 times higher. L-Gulono-1,4-lactone was about ten times more rapidly converted into L-ascorbic acid than the substrate and product of the *myo*-inositol oxygenase-catalyzed reaction. The hen-kidney glucuronolactonase appears to have a K_m-value considerably lower than the values given in the literature for lactonases having other origins[2].

No L-ascorbic acid was synthesized by hen-kidney extracts from *myo*-inositol in the presence of cyanide, although L-ascorbic acid was synthesized rapidly from D-glucurono-6,3-lactone and L-gulono-1,4-lactone. Cyanide is known to inhibit *myo*-inositol oxygenase, which contains nonheme iron[3]. The omission of cyanide, however, decreased the synthesis from D-glucurono-6,3-lactone, and no synthesis was observed from *myo*-inositol. *myo*-Inositol oxygenase was very sensitive to aging, thus resembling the *myo*-inositol oxygenase prepared from rat kidneys[2,3]. The inactivation of the enzyme is probably more rapid in tissue extracts than *in vivo*, which might explain the difference in the experiments with hen-kidney slices and homogenates.

The studies with labelled *myo*-inositol indicate that *myo*-inositol is not consumed to any observable degree by other pathways, since the radioactivity of the *myo*-inositol spot did not decrease significantly during the short incubation times used.

No labelled *myo*-inositol was recovered as labelled L-ascorbic acid when incubated with rat-liver slices. This confirms earlier data concerning the catabolism of *myo*-inositol in the rat.[5,6] The activity of *myo*-inositol oxygenase is high in rat kidney, but very low or absent in liver extracts, which explains the negative results obtained in experiments with tissue slices. On the other hand, rat kidney is not able to synthesize L-ascorbic acid, due to the lack of L-gulono-1,4-lactone oxidase[2].

It appears that the ability to use the *myo*-inositol pathway in the synthesis of L-ascorbic acid is species-specific. The liver is the site of L-ascorbic acid synthesis in mammals and some birds, but the kidneys are the sites in hens and many other birds[7]; the kidneys appear to be the most important organs in *myo*-inositol catabolism in all these species.

A simple method was developed for the lactonase determination. In previous methods, either the decrease of lactone concentration[15] or the increase in gas volume due to CO_2 liberation during the hydrolysis of lactone in hydrogen carbonate buffer[16] are followed. In the former method, it is difficult to saturate the enzyme, and the tissue extracts may contain other enzymes also using D-glucurono-6,3-lactone as substrate, *e.g.*, glucuronolactone dehydrogenase. When the liberation of protons is measured, the enzyme can be saturated. The recording of pH changes is easier to accomplish than the changes in gas volume or pressure and more accurate than to record the additions of minute amounts of base manually or automatically.

ACKNOWLEDGMENT

This study was supported by a grant from the U. S. Public Health Service (AM-06018-02), and from the National Research Council for Natural Sciences, Finland.

REFERENCES

1 S. HOLLMANN AND O. TOUSTER, *Biochim. Biophys. Acta*, 26 (1962) 338.
2 O. HÄNNINEN, *Ann. Acad. Sci. Fenn. Ser.* A, 2, (1968) 142.
3 F. C. CHARALAMPOUS, *J. Biol. Chem.*, 234 (1959) 220.
4 L. V. HANKES, W. M. POLITZER, O. TOUSTER, AND L. ANDERSON, *Ann. N. Y. Acad. Sci.*, 165 (1969) 564.
5 J. J. BURNS, N. TROUSOF, C. EVANS, N. PAPADOPOULOS, AND B. W. AGRANOFF, *Biochim. Biophys. Acta*, 33 (1959) 215.
6 C. H. HOWARD AND L. ANDERSON, *Arch. Biochem. Biophys.*, 118 (1967) 332.
7 S. P. FABRO AND L. M. RINALDINI, *Develop. Biol.*, 11 (1965) 468.
8 O. HÄNNINEN AND J. MARNIEMI, *FEBS Lett.*, 6 (1970) 177.
9 A. G. GORNALL, C. J. BARDAWILL, AND M. M. DAVID, *J. Biol. Chem.*, 177 (1948) 751.
10 R. W. BAILEY, in R. M. C. DAWSON, D. C. ELLIOT, AND K. M. JONES (Eds.), *Data for Biochemical Research*, The Clarendon Press, Oxford, 2nd Edn., 1969, p. 539.
11 H. EAGLE, B. W. AGRANOFF, AND E. E. SNELL, *J. Biol. Chem.*, 235 (1960) 1891.
12 M. K. GAITONDE AND M. GRIFFITHS, *Anal. Biochem.*, 15 (1966) 532.
13 R. M. C. DAWSON AND N. FREUNKEL, *Biochem. J.*, 78 (1961) 606.
14 G. HAUSER, *Ann. N. Y. Acad. Sci.*, 165 (1969) 630.
15 F. EISENBERG, JR., AND J. B. FIELD, *J. Biol. Chem.*, 222 (1956) 293.
16 M. UL HASSAN AND A. L. LEHNINGER, *J. Biol. Chem.*, 223 (1956) 123.