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Transformations of ascorbigen in vivo into ascorbigen acid and 1-deoxy-1-(indol-3-yl)ketoses

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Abstract

The products of the ascorbigen (ASG) in vivo transformation in mice after oral administration were studied by the HPLC method using previously characterized compounds obtained from ASG-ascorbigen acid {2-C-[(indol-3-yl)methyl]-α-L-xylo-3-hexulofuranosonic acid, ASG-acid}, 1-deoxy-1-(indol-3-yl)-α-L-sorbopyranose (Indsorbose) and 1-deoxy-1-(indol-3-yl)-α-L-tagatopyranose (Indtagatose) formed from the ASG-acid after decarboxylation and isomerization. The transformations of ASG were also studied in vitro in phosphate buffer at pH 7.4 or in bovine blood serum; in the latter case, opening of the lactone ring and decarboxylation proceeded faster than in the buffer solution. Invariably Indsorbose and Indtagatose were the major products formed from ASG. The same products were identified in the blood serum and liver of mice, which received ASG per os in a dose of 300 mg/kg. The levels of ASG and its degradation products in the blood serum and in the liver reached a maximum one hour after ASG administration. These data demonstrate that Indsorbose and Indtagatose are the abundant products of the ASG transformations in vivo and are identical to those formed in alkaline media and in serum in vitro.

1. Introduction

Ascorbigen (ASG) is the most abundant indole-derived product in processed cruciferous vegetables, which humans and animals consume with food. It is formed in damaged plants or during food processing from alkaloid glucobrassicin transformation products and L-ascorbic acid (McDanell, McLean, Hanley, Heaney & Fenwick, 1988). Earlier, indole-3-carbinol (I3C) was considered to be the major and the most important indole compound in cruciferous vegetables involved in metabolism of carcinogens (Bjeldanes, Kim, Grose, Bartholomew & Bradfield, 1991; Michnovicz & Bredlow, 1990), and the search for the compound(s) responsible for anticarcinogenic properties of a cruciferous diet was diverted to an inadequate study of chemical and biochemical properties of I3C (Michnovicz & Bredlow, 1990; Preobrazhenskaya & Korolev, 1992). Nowadays it is known that I3C is present in the extracts of cruciferous vegetables only in minor amounts or even traces (Preobrazhenskaya & Korolev; Aleksandrova, Korolev & Preobrazhenskaya, 1992).

ASG is a very reactive compound, which easily undergoes various transformations even in mild acid or alkaline conditions. This suggests that the biological properties of ASG depend to a great extent on the properties of the ASG transformation products, which are to be investigated for understanding the biological properties of ASG. This compound and its analogues undergo transformations of at least two types: those in which L-ascorbic acid is released and the products of the 3-methyleneindolenine oligomerization are formed (Preobrazhenskaya, Korolev, Lazhko, Aleksandrova, Bergman & Lindröm, 1993; Preobrazhenskaya, Bukhman, Korolev & Efimov, 1993), and those in which the transformation of the ascorbic acid backbone of ASG takes place (Scheme 1) (Preobrazhenskaya et al., 1993; Preobrazhenskaya, Lazhko, Korolev, Reznikova & Rozhkov, 1996). The release of L-ascorbic acid and the formation of the 3-methyleneindolenine oligomerization products occur in mild acid conditions including gastric juice (experiments in vitro); the most important products of this process are indolo[3,2-b]carbazole (ICZ) and di(indol-3-yl)methane (DIM), potent ligands of aromatic hydrocarbon responsiveness receptor (Ah

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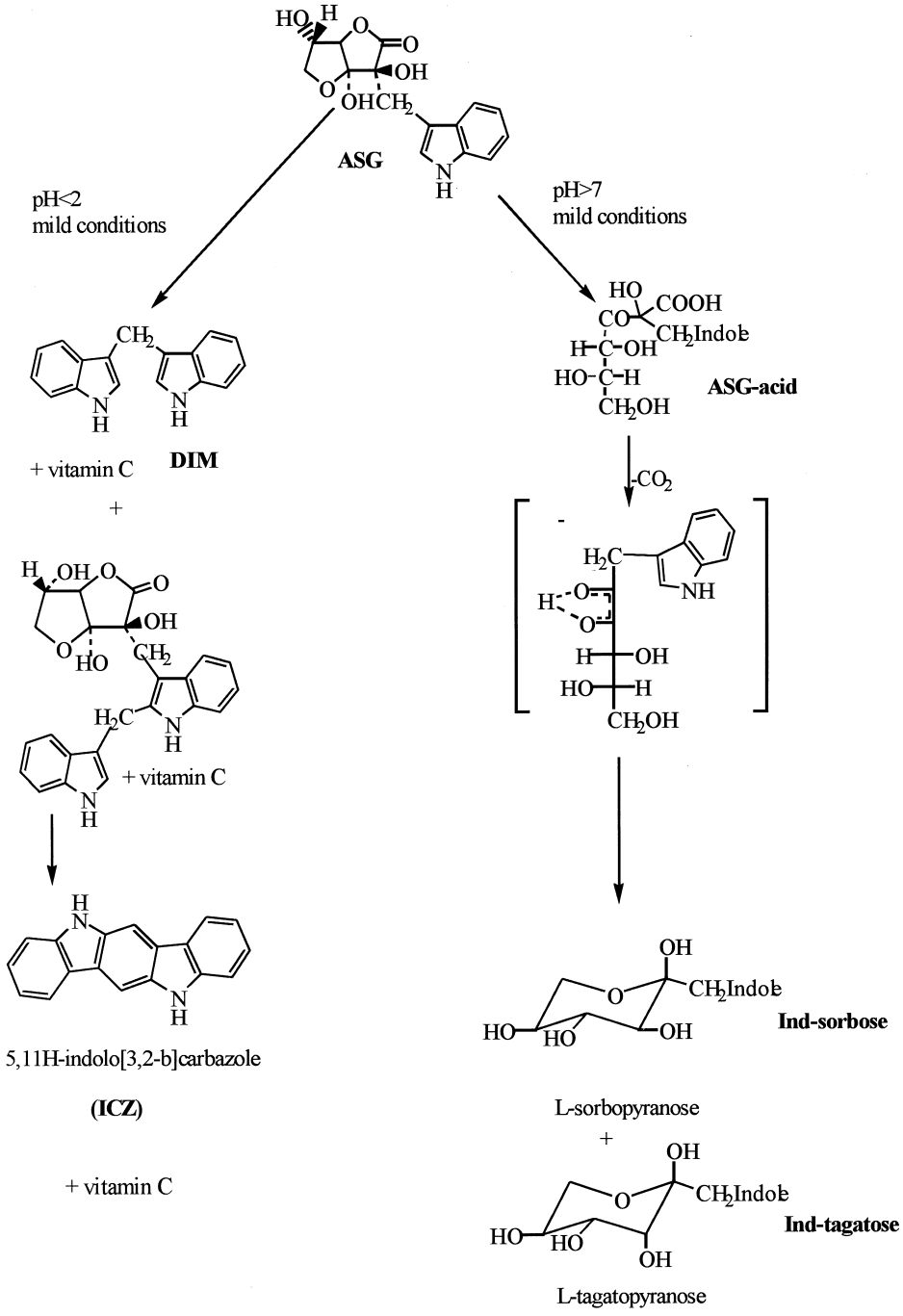

Scheme 1.

receptor) (Preobrazhenskaya, Korolev et al., 1993; Gillner, Bergman, Cambillau, Fernstrom & Gustaffson, 1993). The interaction of ICZ or DIM with Ah receptor leads to the activation of cytochrome P450 1A1-dependent oxidase and in turn to the deactivation of the exogenous carcinogens and also some endogenous compounds, e.g. estradiol (Michnovicz & Bredlow, 1990; Preobrazhenskaya & Korolev, 2000). Thus, ASG is an *in vitro* depot-form of both L-ascorbic acid and ICZ or DIM. Earlier experiments in vivo with humans and guinea pigs demonstrated that only less than 20% of ASG is used by the body as a source of L-ascorbic acid (Matano & Kato, 1967; Virtanen & Kiesvaara, 1963). What happens with the rest and the main portion of ASG (which is not a source of vitamin C) in vivo remains to be investigated.

Earlier it was shown that the transformation of the ascorbic acid backbone of ASG proceeds easily in alkaline media. The opening of the lactone and then furanose rings, the decarboxylation of the resulting β-

keto acid, the isomerization and closing of the pyranose rings to give a mixture of 1-deoxy-1-(indol-3-yl)-α-L-sorbopyranose (Indsorbose) and 1-deoxy-1-(indol-3-yl)-α-L-tagatopyranose (Indtagatose) occur at pH > 7 (Scheme 1). Indtagatose is a stereoisomer of Indsorbose with altered configuration at position 3. The structures of these deoxy ketoses were confirmed by NMR and mass-spectrometry methods. ASG with the opened lactone ring, 2-C-[(indol-3-yl)methyl]-α-L-xylo-3-hexulofuranosonic acid (ASG-acid), was transformed into the corresponding diphenylmethyl ester whose structure was also confirmed by NMR and mass-spectrometry (Preobrazhenskaya et al., 1996).

The goal of this project was the investigation of the ASG transformation products in vivo in the blood and tissues of mice which obtained ASG per os using products of the ASG chemical transformations as standards.

2. Materials and methods

2.1. Transformation of the ascorbigen in model systems in vitro

ASG (0.3–1.5 μM) was incubated in 0.1 M phosphate buffer, pH 7.4, bovine blood serum, or mouse liver homogenate; the samples were stored at 37°C, and then ASG and the products formed were prepared for HPLC analysis.

2.2. Preparation of ASG transformation products for HPLC analysis

An aliquot of the incubation mixture was mixed with a double volume of a mixture phosphate buffer, pH 5.3: ethanol (v/v, 1:1); (stop buffer), then ethanol was added in a double volume relative to the aliquot. After each addition, the mixture was stirred vigorously. The samples containing bovine blood serum or a homogenate were centrifuged in an angular rotor at 800 g for 30 min. The supernatant was injected into the chromatograph without dilution. In experiments with the phosphate buffer, the samples obtained after the incubation were injected into HPLC chromatograph.

HPLC analyses were performed on a Shimadzu HPLC instrument of the LC 10 series. Analytical reverse phase HPLC was carried out on a Diasorb C16 column (particle size 7 μm) at an injection volume of 10 μl and a wavelength 280 nm. The sample concentration was 0.05–0.2 mg/ml. The system comprised 0.01 M H_3PO_4, pH 2.6 (A) and acetonitrile (B). The proportion of acetonitrile varied from 10 to 45% for 17 min and then from 45 to 80% for 8 min with flow rate 1.0 ml/min (system 1), or from 7 to 10% for 20 min and then from 10 to 45% for 10 min with flow rate 1.3 ml/min (system 2). The retention times of ASG and its transformation products are presented in Table 1. The bovine blood serum or liver homogenate extracts were separated in system 2, and all other samples in system 1.

2.3. In vivo experiments

Male mice $F_1(CBA \times C_{57}BL_{j6})$ weighing 22–24 g, from the animal house Kriukovo of the Russian Academy of Medical Sciences and fed on the standard laboratory diet, were used in the experiments. ASG was introduced per os at a dose of 300 mg/kg in water solution (total dose volume 0.4 ml). After 1, 2 or 3 h the animals were sacrificed by cervical dislocation, and the blood, liver, stomach and intestine were taken. The blood was centrifuged in an angular rotor at 200 *g* for 8 min. The liver and other organs were frozen, weighed and homogenized in a double volume of 0.1 M phosphate buffer (pH 5.3):ethanol mixture (v/v, 1:1). To the aliquots of homogenates, a double volume of ethanol was added, and the mixture was stirred, and centrifuged at 800 *g* for 30 min. To the aliquots of serum, a double volume of the phosphate buffer (pH 5.3) and ethanol (v/v, 1:1) mixture and then a double volume of ethanol were added, the mixture was stirred and then centrifuged.

2.4. Liver homogenate for in vitro investigation

The mice that did not obtain ASG were sacrificed and the liver was taken out, frozen, weighed and homogenized in a Biomix homogenizer in a triple volume of 0.1 M phosphate buffer at pH 7.4.

3. Results and discussion

At the first stage of study, ASG was incubated in the phosphate buffer, pH 7.4, or in the bovine blood serum and the ASG transformation products studied. The HPLC profile of the products formed from ASG in the bovine blood serum after 1 h of incubation at 37°C is presented in Fig. 1. The main products here are ASG-acid (ASG with the open lactone ring), Indsorbose, and Indtagatose. It should be mentioned that ASG-acid and ASG are in equilibrium, and the ratio of these products in HPLC largely depends on the stop buffer used before the isolation of the degradation products (a phosphate buffer with pH 5.3 was used), though opening of the

Table 1
Retention times (min) of the compounds investigated

System	Indtagatose	ASG-acid	Indsorbose	ASG	Indol-3-ylacetic acid
1	6.8	7.9	8.6	10.8	14.2
2	14.1	17.8	19.8	23.6	27.4

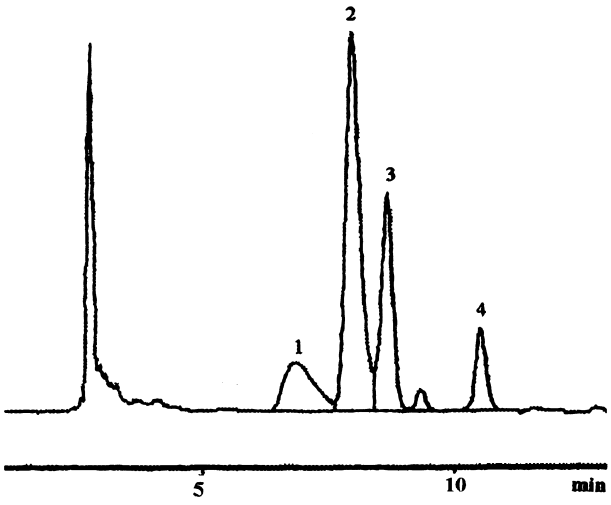

Fig. 1. HPLC (system 1) analysis of the ASG transformation products after incubation in bovine blood serum at 37°C for 1 h. (1) Indtagatose; (2) ASG-acid; (3) Indsorbose; (4) ASG.

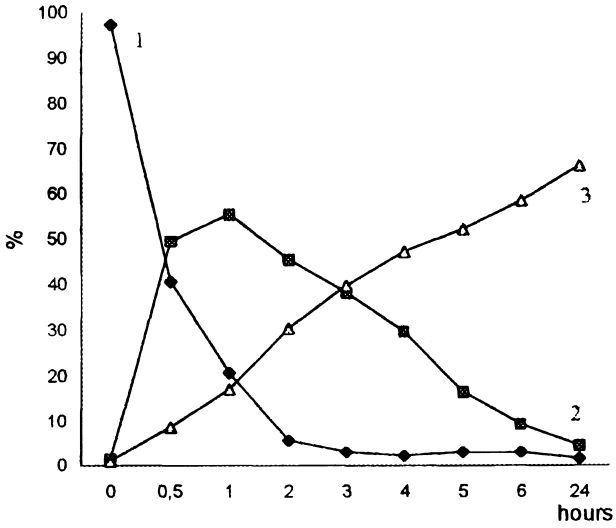

Fig. 2. Transformation of ASG incubated in phosphate buffer at pH 7.4 at 37°C. (1) ASG; (2) ASG-acid; (3) Indsorbose + Indtagatose.

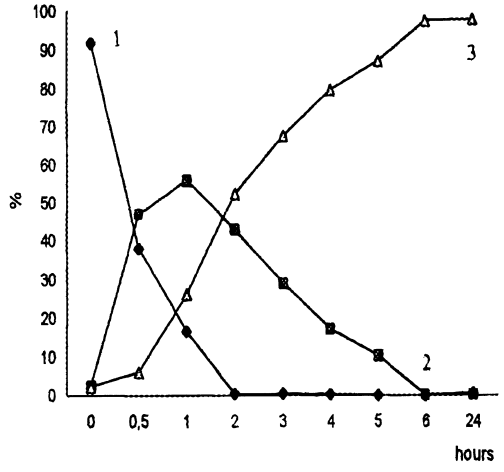

Fig. 3. Transformation of ASG incubated in bovine blood serum at 37°C. (1) ASG; (2) ASG-acid; (3) Indsorbose + Indtagatose.

ASG lactone ring proceeds rather slowly and takes about 2 h in serum (Fig. 2). The transformations of ASG in the phosphate buffer and the changes in the ASG, ASG-acid, and Indketoses concentrations are presented in Fig. 3. It can be seen that the accumulation of ASG-acid takes place first and this compound then decarboxylates to afford ketoses, the major final incubation products. In the serum, the transformation of ASG proceeds faster than in the buffer (see Fig. 4): within 6 h, almost 100% of ASG was transformed into ketoses in serum, and only about 60% in buffer (Fig. 4).

The blood serum of mice, to which ASG was given per os in a dose of 300 mg/kg, contains the same products that were formed in buffer or in blood serum in vitro (Fig. 5). One hour after the ASG administration, ASG and ASG-acid are still present in blood serum as well as Indsorbose, Indketose and an unidentified compound. A preliminary study of the ASG transformation products, after different periods, following the per os

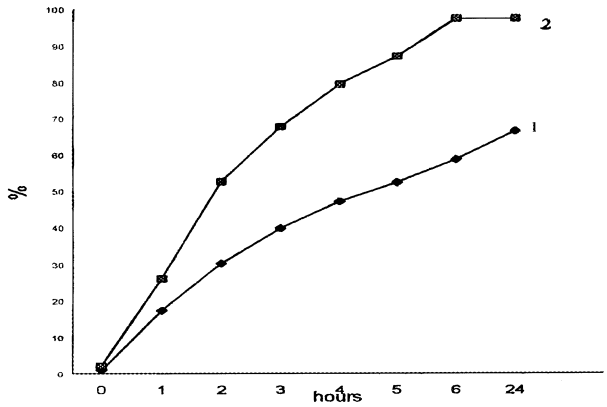

Fig. 4. Kinetics of the indolyl ketoses (Indsorbose + Indtagatose) formation in (1) phosphate buffer at pH 7.4 and (2) in bovine blood serum at 37°C.

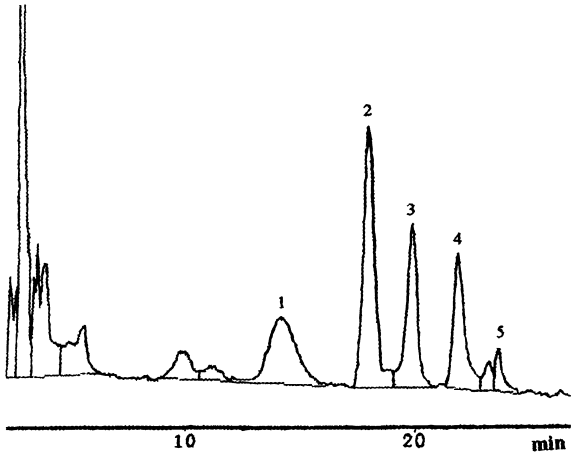

Fig. 5. HPLC analysis (system 2) of blood serum extract of mice, to which ASG was given per os in the dose of 300 mg/kg 1 h previously. (1) Indtagatose; (2) ASG-acid; (3) Indsorbose; (4) unidentified compound; (5) ASG.

administration of ASG to mice, showed that, after 1 h, ASG almost totally disappeared from the stomach and intestine and the level of ASG and the products of its degradation in blood and liver reached a maximum (in stomach and intestine the unchanged ASG predominates whereas its transformation products are present only in traces). After 1 h, the levels of these compounds start decreasing. In Fig. 6, the ASG-derived compounds determined in the liver of mice to which ASG had been administered per os (300 mg/kg) are presented.

The levels of Indsorbose and Indtagatose in extracts of the liver homogenate are comparable with the level of these compounds in blood serum. The ASG-acid content is thereby lower than in blood, which means that the ASG and ASG-acid equilibrium is shifted toward ASG. This may be related to the pH of the liver homogenate (Fig. 6).

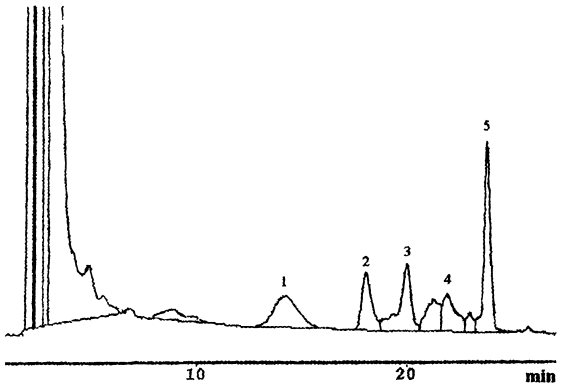

Fig. 6. HPLC analysis (system 2) of liver homogenate extract of mice, to which ASG was given per os in the dose of 300 mg/kg 1 h previously. (1) Indtagatose; (2) ASG-acid; (3) Indsorbose; (4) unidentified compound; (5) ASG.

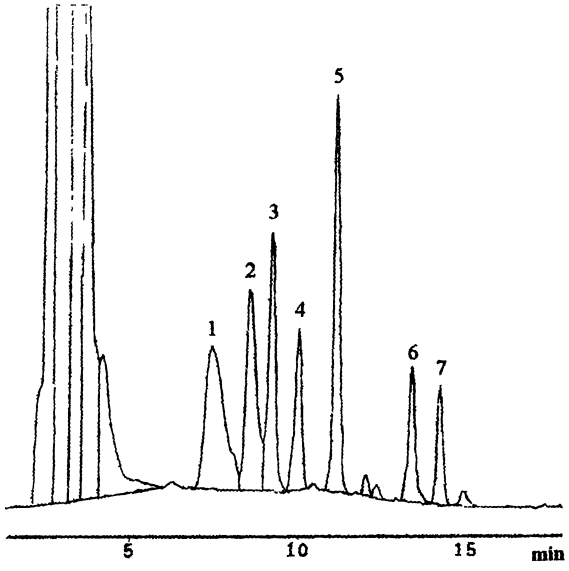

Fig. 7. HPLC analysis (system 1) of the products of ASG transformation after incubation of ASG in mice liver homogenate for 2 h at 37°C. (1) Indtagatose; (2) ASG-acid; (3) Indsorbose; (4) unidentified compound; (5) ASG; (6) indole-3-acetic acid; (7) unidentified compound.

In Fig. 7, the HPLC profile of the extracts of products formed from ASG in the liver homogenate, in vitro after 2 h of incubation, is presented. The goal of this experiment was to investigate the possibility of enzymatic transformations of ASG and related compounds. In addition to ASG-acid and ketoses, a peak of indol-3-ylacetic acid (IAA) is present here. Possibly, IAA is formed in the degradation processes of ASG-acid or ketoses. However, IAA was not identified among products formed in vivo in liver. This suggests that IAA undergoes further in vivo transformations or its concentration in vivo was too low to be detected by the methods used.

It is known that ASG in acidic media releases L-ascorbic acid and produces ICZ and other methyleneindolenine oligomerization products, which are not seen in the HPLC systems used in the present study. However, the process of the vitamin C release and methyleneindolenine oligomerization products (including ICZ) formation is not fast (in any case, in experiments in vitro) (Preobrazhenskaya, Korolev et al., 1993) and our preliminary experiments did not reveal the presence of ICZ in stomach and its contents 1 h after per os administration of ASG.

These data demonstrate that Indsorbose and Indketose are the most abundant products of the ASG transformations in vivo. In any case, they suggest that a large portion of the introduced ASG is not involved in the production of vitamin C or ICZ.

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