Regulation of Ascorbic Acid and of Xylulose Synthesis in Rat-Liver Extracts

THE EFFECT OF STARVATION ON THE ENZYMES OF THE GLUCURONIC ACID PATHWAY

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1. The synthesis of ascorbic acid in rat-liver extracts is impaired during starvation, and more from glucuronolactone and glucuronate than from gulonate and gulonolactone. 2. The formation of xylulose from gulonate and from gulonolactone is greatly enhanced during starvation, whereas it is decreased from glucuronolactone and from glucuronate. 3. The activity of the enzymes of the glucuronic acid pathway during starvation has been determined in rat-liver preparations. Gulonolactone oxidase is decreased, NAD-linked gulonate dehydrogenase is enhanced, and uronolactonase, aldolactonase and NADP-linked hexonate dehydrogenase are unchanged. 4. The impairment of ascorbic acid synthesis from gulonate observed during starvation can be accounted for by the depressed activity of gulonolactone oxidase. 5. The cause of the enhanced formation of xylulose has been located in the sedimentable fraction of liver homogenate. 6. The hypothesis is formulated of an increased utilization of the glucuronic acid pathway during starvation.

The synthesis of ascorbic acid from D-glucuronolactone in rat-liver extracts is greatly decreased during starvation or short-term deprivation of dietary carbohydrates (Stirpe, Comporti & Caprino, 1963). This impairment, which was interpreted on the basis of an enzymic adaptation, could be caused by decreased activity of some enzyme(s) in the sequence of reactions leading from the substrate to the product, or by removal of the substrate or of some intermediate(s) formed. It has also been observed (Stirpe & Comporti, 1963) that these conditions causing an impairment of ascorbic acid synthesis induced also an increased formation of L-xylulose from L-gulonate. Since the latter compound is an intermediate in the synthesis of ascorbic acid, the hypothesis was formulated that the impairment of ascorbic acid synthesis could be due at least in part to the removal of gulonate to form xylulose.

The present paper describes a survey of the activity of the enzymes of the pathways leading from glucuronolactone to ascorbic acid and to xylulose. The investigation was limited to the effects of starvation, which were more marked than those of carbohydrate deprivation. Results indicated that in starved rats the activity of the microsomal gulonolactone oxidase is somewhat decreased, and confirmed the enhanced formation of xylulose from

gulonate. They indicated also that the increased formation of xylulose and the impaired synthesis of ascorbic acid, although being two simultaneous effects of starvation, might be independent from each other.

Information was also obtained on the normal operation of the pathways studied.

EXPERIMENTAL

Chemicals. L-Gulonolactone and NADPH were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Potassium salts of D-glucuronic acid and L-gulonic acid were prepared from the corresponding lactones, as described by Hach & Benjamin (1954) and by Bublitz & Lehninger (1961) respectively. L- and D-Xylulose were a generous gift from Dr G. Ashwell, Bethesda, Md., U.S.A. All other chemicals were obtained from the sources indicated by Stirpe *et al.* (1963).

Animals. Male rats of the Wistar Glaxo strain weighing 140–160 g. were used. In the experiments with the subfractions a larger amount of liver was necessary, and rats weighing 200 g. were used. The general conditions of housing and handling were the same as those given by Stirpe *et al.* (1963). The animals were fed on the stock diet described by Bonetti & Stirpe (1962), given to rats mixed with 15% of water.

Preparations of liver extracts and subfractions. Rats were stunned with a blow on the head and exsanguinated by decapitation. The liver was quickly excised, washed with ice-cold 0.15 M-KCl and weighed. In most experiments the

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liver was homogenized in 0.15 M-KCl (1g. of liver + 2.5 ml. of KCl); the homogenate was centrifuged for 10 min. at 3000g at 0° in a Martin-Christ Universal Junior IIKS refrigerated centrifuge fitted with multi-speed attachment and rotor no. 5070. In other experiments the homogenate was prepared in sucrose (1g. of liver + 4 ml. of 0.25 M-sucrose) and centrifuged for 10 min. at 9000–10000g in the same centrifuge. In either case the supernatant was used, and is referred to below as 'KCl extract' or 'sucrose extract', as appropriate.

In some experiments these extracts were fractionated further by centrifugation at 86000g at 0° in a Martin-Christ Omikron centrifuge with rotor no. 288 for 40 min., including the time for acceleration (about 10 min.) but not that for stopping (12–15 min.). A 'KCl sediment' and a 'KCl supernatant' were obtained from the KCl extracts; the fractions obtained from the sucrose extracts are referred to below as 'microsomal fraction' and 'sucrose supernatant'. Unless otherwise stated, the tube walls and the surface of the KCl sediment and of the microsomal fraction were washed with a few millilitres of KCl or sucrose solution respectively, and the pellets resuspended in the same medium in such a way as to have the product from 200 mg. of liver in 1 ml.

For the experiments on NAD-linked gulonate dehydrogenase and on NADP-linked hexonate dehydrogenase the liver was made free of blood by perfusing it *in situ*, after the animal had been killed, with 20–40 ml. of ice-cold 0.15 M-KCl injected through the inferior vena cava. Homogenates for these experiments were prepared with 0.25 M-sucrose (1g. of perfused liver + 9 ml. of sucrose), and were centrifuged at 10000 and 86000g as described above; the final supernatant was used for the enzymic assays.

All operations were carried out as close to 0° as possible, and the preparations obtained were used with minimum delay.

Synthesis and destruction of ascorbic acid. The synthesis of ascorbic acid was studied in a system with KCl or sucrose extracts corresponding to 200 mg. of liver, with D-glucuronolactone, potassium D-glucuronate, potassium L-gulonate or L-gulonolactone as substrates, and with the additions and cofactors described by Stirpe *et al.* (1963). In preliminary experiments optimum conditions for the synthesis of ascorbic acid from each substrate were determined, with special consideration for linearity with time and quantity of extract. The mixtures were incubated at 37° in a Dubnoff shaker, with air as gas phase, for 2 hr. when glucuronolactone or glucuronate were substrates, and for 1 hr. with gulonate or gulonolactone.

Destruction of ascorbic acid was studied in a similar system, with 1 μ mole of ascorbic acid as substrate.

Synthesis and destruction of xylulose. Xylulose formation was determined in the same mixtures incubated for the synthesis of ascorbic acid; values obtained in blanks without substrate were subtracted. Destruction was studied by substituting 20 μ moles of L-xylulose for the substrate in the same system.

Uronolactonase (D-glucurono- δ -lactone hydrolase, EC 3.1.1.19) and **aldonolactonase** (L-gulono- γ -lactone hydrolase, EC 3.1.1.18). The activity of these enzymes was assayed in the KCl extracts by measuring the disappearance of 10 μ moles of glucuronolactone and gulonolactone respectively from the incubation mixtures used for ascorbic acid synthesis.

Gulonolactone oxidase (L-gulono- γ -lactone-oxygen oxidoreductase, EC 1.1.3.-). This was assayed essentially according to the method of Kanfer, Burns & Ashwell (1959), by determining the amount of ascorbic acid formed by microsomal fraction corresponding to 200 mg. of liver, incubated for 60 min. in the same medium used for the synthesis of ascorbic acid with liver extracts.

NADP-linked hexonate dehydrogenase (L-gulonate-NADP oxidoreductase, EC 1.1.1.19, and **NAD-linked gulonate dehydrogenase** (3-hydroxy acid-NAD oxidoreductase, EC 1.1.1.45). NADP-linked hexonate-dehydrogenase activity was measured by following the oxidation of NADPH, as described by Salomon & Stubbs (1961), with potassium glucuronate as substrate.

NAD-linked gulonate-dehydrogenase activity was determined by following the reduction of NAD, as described by Smiley & Ashwell (1961), with potassium gulonate as substrate.

Sucrose supernatant from perfused liver was used for these determinations. It was observed that the activity of these enzymes was greatly influenced by the temperature. Since a thermostatically controlled spectrophotometer was not available, the considerable variations of room temperature were overcome by running the reaction in the spectrophotometer cuvettes immersed in a water bath at 37°, from which they were removed immediately before measuring the extinction at 5 and 10 min. after the addition of supernatant. Variations in the absence of substrate were subtracted: they were very small for NAD and almost nil for NADPH.

The oxidation or reduction of nicotinamide nucleotide coenzymes was followed by estimating the changes of E_{340} in a Uvispek spectrophotometer (quartz cell). A molar extinction coefficient 6.22 was assumed in calculations.

Chemical determinations. Ascorbic acid was determined by the method of Roe & Kuether (1943), as described by Stirpe *et al.* (1963).

Xylulose was determined by the colorimetric method of Kulka (1956), a standard curve being run with each experiment with samples containing known amounts of D-xylulose and the same quantity of trichloroacetic acid as the samples from the incubated mixtures. Xylulose was identified by the enzymic tests with NAD- and NADP-linked xylitol dehydrogenases (Hickman & Ashwell, 1959).

Lactones were determined by the method of Lipmann & Tuttle (1945). Optimum development of purple colour was obtained by using 0.3 ml. of HCl diluted 1:4 (v/v) instead of 1 ml. as used in the original method.

Nitrogen was determined by the micro-Kjeldhal method on diluted samples of each preparation used.

For all colorimetric determinations a Unicam SP.600 spectrophotometer was used.

RESULTS

Two schemes of reactions have been postulated to explain the conversion of glucuronolactone into ascorbic acid: according to that proposed by Isherwood, Chen & Mapson (1954) glucuronolactone is hydrolysed to glucuronic acid, and this is converted into gulonic acid, gulonolactone and ascorbic acid. According to Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1960a) and to Yamada, Suzuki, Mano &

Table 1. *Recovery of ascorbic acid incubated with liver extracts*

Incubation mixtures contained in a final volume of 2.5 ml.: 50 μ moles of potassium phosphate buffer, pH 7.4; 4 μ moles of NAD; 4 μ moles of ATP; 75 μ moles of nicotinamide; 5 μ moles of $MgCl_2$; 1 μ mole of L-ascorbic acid; 0.7 ml. of KCl extract.

Expt. no.	Conditions	Ascorbic acid recovered after 2 hr. (μ mole)
1	Normal rat, complete medium	0.965
	Starved rat, complete medium	0.960
2	Normal rat, complete medium	0.915
	Normal rat, NAD and ATP omitted	0.392
	Starved rat, complete medium	0.905

Fig. 1. Disappearance of glucuronolactone and formation of ascorbic acid in rat-liver extracts. Incubation mixtures were as described in Table 1, with 10 μ moles of glucuronolactone as substrate. ●, Glucuronolactone, normal rat; ○, glucuronolactone, starved rat; ▲, ascorbic acid, normal rat.

Shimazono (1962) glucuronolactone may be converted directly into gulonolactone. Both possibilities have been considered in the planning of the experiments described below.

Destruction of ascorbic acid. To ascertain whether an accelerated destruction rather than a decreased synthesis of ascorbic acid occurred in liver extracts from starved rats, the disappearance of ascorbic acid added to the incubation mixtures was determined. The ascorbic acid was recovered almost quantitatively at the end of the incubation, and no differences were observed between normal and starved rats (Table 1). It was concluded from these experiments that a real impairment of ascorbic acid synthesis occurred in rat liver during starvation. A relevant

Fig. 2. NADP-linked hexonate-dehydrogenase activity of liver supernatant from normal and starved rats. Results are given as mean values of four experiments per group (the s.e.m. is not shown because it is narrower than experimental points). The reaction mixture contained, in a final volume of 3 ml.: 150 μ moles of tris-HCl buffer, pH 7.6; 30 μ moles of KCl; 30 μ moles of nicotinamide; 9 μ moles of potassium glucuronate or glucuronolactone; 0.3 μ mole of NADPH; 0.2 ml. of sucrose supernatant from perfused rat liver. ●, Glucuronate, normal rats; ○, glucuronate, starved rats; ▲, glucuronolactone, normal rats.

destruction of ascorbic acid occurred if ATP and NAD were omitted from the incubation medium.

Uronolactonase and aldonolactonase. The uronolactonase activity of liver extracts was very high. Typical results of a time-course study of lactone disappearance in parallel with ascorbic acid formation are reported in Fig. 1. The lactone disappears almost completely in 20–30 min. A 'lag-phase' exists in the formation of ascorbic acid, this phase terminating approximately when the glucuronolactone has disappeared. Disappearance of gulonolactone is even faster, being almost complete in about 5 min., and therefore these results are not shown.

The activity of both lactonases is of the same order in extracts from normal as well as from starved rats; a representative result of uronolactonase activity during starvation is given in Fig. 1. From these results it was concluded that neither of the two lactonases could be the limiting factor for the synthesis of ascorbic acid in starved rats.

NADP-linked hexonate dehydrogenase. This enzyme is located in the soluble supernatant in guinea-pig (Ishikawa & Noguchi, 1957) and in rat liver (Mano, Suzuki, Yamada & Shimazono, 1961). According to York, Grollman & Bublitz (1961) this enzyme is inactive on glucuronolactone, and according to ul Hassan & Lehninger (1956) is more active on glucuronate, but according to Mano, Yamada, Suzuki & Shimazono (1959) and to Mano *et al.* (1961) it is more active on glucuronolactone. This was investigated, and it was observed that the enzymic activity of the sucrose supernatant with glucuronate was about double that with glucuronolactone as substrate (Fig. 2), and consequently the former substrate was used for the assays. The hexonate-dehydrogenase activity was not affected by starvation.

Gulonolactone oxidase. This enzyme was the last one to be examined in the pathway leading from glucuronolactone to ascorbic acid. Gulonolactone oxidase has been located in the microsomal fraction (Kanter *et al.* 1959; Bublitz & Lehninger, 1959; Chatterjee *et al.* 1960a). Since the impairment of ascorbic acid synthesis from glucuronolactone had been observed with potassium chloride extracts, the potassium chloride sediment was used for the oxidation of gulonolactone in a first set of experiments. It was noticed that the pellet obtained by high-speed centrifugation of potassium chloride extracts consisted of two layers, whose proportions varied with the nutritional status of the animals, the bottom layer being more abundant in normal rats. It may be assumed from the centrifugation data that the potassium chloride sediment consists mainly of microsomes contaminated by mitochondria and, in fed rats, by glycogen (Siekevitz, 1962). The results of the enzymic determinations could be misinterpreted if the proportions of nitrogen-containing contaminating matter were different under different dietary conditions. To avoid this, the gulonolactone oxidase was determined with microsomal preparations obtained with the usual technique from sucrose homogenates, after it had been checked that the synthesis of ascorbic acid from glucuronolactone was impaired during starvation in sucrose extracts as much as in the potassium chloride extracts.

The gulonolactone-oxidase activity of microsomal preparations was determined in parallel with the synthesis of ascorbic acid from gulonolactone in sucrose extracts (Table 2). The gulonolactone-oxidase activity was lowered in starved rats by a similar extent (30%) to the synthesis of ascorbic acid in sucrose extracts (35%). However, the results obtained with microsomes were very variable, and only the synthesis of ascorbic acid in sucrose extracts from starved rats was statistically significantly different from normal.

The complete medium with NAD, ATP etc. was

Table 2. *Formation of ascorbic acid by microsomal fraction (gulonolactone oxidase) and by sucrose extracts*

Experimental conditions were as given in Table 1, but with 20 μ moles of gulonolactone as substrate and 1 ml. of microsomal fraction or of sucrose extract, and incubation was for 1 hr. Results are given as mean values $\pm$ s.e.m. of six experiments in each group.

Treatment	Ascorbic acid formed (μ moles/mg. of N/hr.)	
	Microsomal fraction	Sucrose extract
Fed	1.246 $\pm$ 0.150	0.225 $\pm$ 0.014
Starved	0.869 $\pm$ 0.152*	0.147 $\pm$ 0.005†

* Statistically not different from fed group (0.1 > P > 0.05).

† Statistically different from fed group (P < 0.001).

used in these and in the following experiments for studying the synthesis of ascorbic acid from gulonate or gulonolactone, although the lactonization and subsequent oxidation do not require any of these cofactors. However, a higher yield of ascorbic acid was obtained when they were present in the incubation mixture. It is likely that these cofactors act by preventing destruction of the ascorbic acid formed (see Table 1), since less ascorbic acid was found after 2 hr. than after 1 hr. of incubation when they were omitted (0.652 and 0.955 μ mole of ascorbic acid formed/200 mg. wet wt. of liver after 1 and 2 hr. of incubation respectively, against 0.555 and 0.425 μ mole/200 mg. respectively in the medium without cofactors).

Formation of xylulose. The hypothesis has been mentioned that the impairment of ascorbic acid synthesis could be due to removal of the substrate or of an intermediate. A possibility for the operation of this mechanism would be the removal of gulonate to form xylulose, if the activity of NAD-linked gulonate dehydrogenase or 3-oxogulonate decarboxylase or both were enhanced by starvation. This working hypothesis was tested by determining the formation of xylulose from gulonate in potassium chloride extracts, in parallel with the synthesis of ascorbic acid. The decrease in ascorbic acid synthesis was actually accompanied by increased formation of xylulose (Stirpe & Comporti, 1963). Xylulose was identified enzymically as the L-form.

The formation of ascorbic acid and of xylulose from the substrates of the glucuronic acid pathway is summarized in Table 3. In normal rats the amount of ascorbic acid formed increases, in the order given, from glucuronolactone to glucuronate, gulonate and gulonolactone. The synthesis of ascorbic acid from glucuronolactone and from glucuronate in starved rats is greatly impaired. The im-

Table 3. *Synthesis of ascorbic acid and of xylulose from various substrates in normal and starved rats*

Experimental conditions were as given in Tables 1 and 2, with 20 μ moles of substrate and 0.7 ml. of KCl extract. Results are given as mean values $\pm$ s.e.m., with the numbers of experiments in parentheses.

Substrate ... Treatment	Ascorbic acid formed			
	$(\mu\text{mole/mg. of N/2hr.})$		$(\mu\text{mole/mg. of N/hr.})$	
	Glucuronolactone	Glucuronate	Gulonate	Gulonolactone
Fed	0.185 $\pm$ 0.027 (6)	0.212 $\pm$ 0.024 (6)	0.192 $\pm$ 0.007 (18)	0.232 $\pm$ 0.018 (10)
Starved	0.025 $\pm$ 0.004 (11)	0.023 $\pm$ 0.003 (11)	0.127 $\pm$ 0.004 (23)	0.164 $\pm$ 0.008 (15)
Substrate ... Treatment	Xylulose formed			
	$(\mu\text{moles/mg. of N/2 hr.})$		$(\mu\text{moles/mg. of N/hr.})$	
	Glucuronolactone	Glucuronate	Gulonate	Gulonolactone
Fed	2.21 $\pm$ 0.29 (5)	2.26 $\pm$ 0.17 (6)	0.97 $\pm$ 0.08 (16)	0.92 $\pm$ 0.07 (10)
Starved	1.68 $\pm$ 0.04 (7)	1.27 $\pm$ 0.06 (7)	2.38 $\pm$ 0.17 (17)	2.02 $\pm$ 0.16 (11)

Table 4. *Destruction of L-xylulose by liver extracts of normal and starved rats*

Experimental conditions were as given in Table 3, with 20 μ moles of L-xylulose or of potassium gulonate as substrate. The synthesis of ascorbic acid and of xylulose in the same extracts was determined to check the effect of starvation.

Treatment	Xylulose destroyed $(\mu\text{mole/mg. of N/hr.})$	Ascorbic acid formed $(\mu\text{mole/mg. of N/hr.})$	Xylulose formed $(\mu\text{moles/mg. of N/hr.})$
Fed	0.59	0.172	1.52
Starved	0.53	0.137	2.41

Table 5. *Formation of ascorbic acid and of xylulose by potassium chloride extracts with and without NAD*

Experimental conditions were as given in Table 3, with 20 μ moles of potassium gulonate as substrate. Results are given as mean values of three experiments for each group.

Treatment	Ascorbic acid formed $(\mu\text{mole/mg. of N/hr.})$		Xylulose formed $(\mu\text{moles/mg. of N/hr.})$	
	With NAD	Without NAD	With NAD	Without NAD
Fed	0.153	0.173	1.02	0.21
Starved	0.109	0.122	3.26	0.45

pairment of ascorbic acid synthesis from gulonate and from gulonolactone is substantially lower. The formation of xylulose from gulonate or from gulonolactone in starved rats is greatly enhanced (almost equally), whereas the amount of xylulose formed from glucuronolactone or glucuronate is decreased during starvation.

The accumulation of xylulose in extracts from starved rats was due to increased formation and not to lowered destruction (Table 4). This enhanced formation of xylulose could account in part for the impaired synthesis of ascorbic acid from gulonate and gulonolactone, or could be a purely coincident phenomenon. To ascertain this, experiments were performed in which the formation of ascorbate as well as of xylulose was studied with and without

NAD in the reaction medium. Since gulonate dehydrogenase is NAD-dependent, formation of xylulose was abolished when NAD was omitted. The synthesis of ascorbic acid was impaired at the same extent in starved rats even when formation of xylulose was hindered by the omission of NAD (Table 5).

Gulonate dehydrogenase and the pathway to xylulose. The conversion of gulonate into xylulose is catalysed by gulonate dehydrogenase and 3-oxogulonate decarboxylase (3-oxo-L-gulonate carboxy-lyase, EC 4.1.1.-). Gulonate dehydrogenase is located in the soluble supernatant (Chatterjee *et al.* 1960a; Isherwood, Mapson & Chen, 1960; Smiley & Ashwell, 1961). The activity of sucrose supernatant preparations was about 30% higher in starved than in

normal rats (0.970 ± 0.05 and $0.761 \pm 0.06 \mu\text{mole}$ of NAD reduced/mg. of N/10 min. respectively), which is much less than the enhancement observed in the formation of xylulose.

An obstacle to the estimation of 3-oxogulonate decarboxylase was the difficult preparation and the limited stability of the substrate (Winkelman & Ashwell, 1961). It was hoped to obtain at least some information on the activity of this enzyme by comparing the reduction of NAD with the amount of xylulose formed. The decarboxylase has been purified from preparations consisting probably of supernatant with some particulate matter (Winkelman & Ashwell, 1961), but formation of xylulose has been obtained with preparations from soluble supernatant (Chatterjee *et al.* 1960*a*; Isherwood *et al.* 1960)

and therefore the potassium chloride supernatant was used for the present experiments. However, it was observed that no xylulose was formed from gulonate when the potassium chloride supernatant was incubated with the same medium used for studying the synthesis of xylulose with potassium chloride extracts. This led us to investigate which subcellular fraction had to be present to obtain xylulose formation. Both sediment and supernatant were necessary, and heating of either fraction in a boiling-water bath for 5 min. prevented xylulose formation (Table 6).

The synthesis of ascorbic acid and of xylulose was studied in mixtures of potassium chloride sediment from a starved rat with potassium chloride supernatant from a normal one and *vice versa*. In these experiments the sedimented pellets were not washed after decanting the supernatant (see the Experimental section) to avoid loss of particulate matter. Results were compared with those obtained with recombined sediment and supernatant from the same rat (Table 7). The amount of xylulose formed in mixtures of potassium chloride sediment from starved rats and of supernatant from normal ones was well above that obtained with the recombined fractions from normal rats and very close to the quantity formed by the recombined fractions from starved rats. The addition of supernatant from starved rats to the sediment fraction from normal rats did not enhance xylulose formation beyond the amount formed by the recombined fractions from normal rats. The quantity of ascorbic acid formed by the mixture of sediment from normal rats and supernatant from starved rats was very close to that formed by the recombined fractions from normal rats, account being taken of the deterioration due to fractionation and recombination. Ascorbic acid

Table 6. *Formation of xylulose by potassium chloride extracts or subfractions*

Experimental conditions were as described in Table 3, with 20 μmoles of gulonate as substrate and 0.7 ml. of KCl extract or subfractions (corresponding to 200 mg. of liver).

Expt. no.	Preparation used	Xylulose formed ($\mu\text{moles/mg. of N/hr.}$)	
		Normal rat	Starved rat
1	Unfractionated extract	1.11	2.59
	Sediment	0.39	0.38
	Supernatant	0.09	Traces
2	Sediment + supernatant (recombined)	1.87	3.23
	Sediment + boiled supernatant	0.53	Not determined
	Supernatant + boiled sediment	0.11	0.19

Table 7. *Synthesis of ascorbic acid and of xylulose by potassium chloride extracts and recombined subfractions from normal and starved rats*

Experimental conditions were as given in Table 3, with 20 μmoles of potassium gulonate as substrate. Results are given as mean values $\pm$ S.E.M. of four experiments for each group.

Preparation used		Ascorbic acid formed		Xylulose formed	
		($\mu\text{mole/200 mg. wet wt./hr.}$)	($\mu\text{mole/mg. of N/hr.}$)	($\mu\text{moles/200 mg. wet wt./hr.}$)	($\mu\text{moles/mg. of N/hr.}$)
Sediment	Supernatant				
Normal (unfractionated)	Normal	0.654 ± 0.089	0.168 ± 0.012	3.87 ± 0.61	0.99 ± 0.14
Normal (recombined)	Normal	0.573 ± 0.060	0.148 ± 0.017	4.42 ± 0.11	1.13 ± 0.27
Starved (unfractionated)	Starved	0.390 ± 0.034	0.100 ± 0.008	11.31 ± 0.93	2.87 ± 0.13
Starved (recombined)	Starved	0.393 ± 0.032	0.099 ± 0.008	11.65 ± 0.75	2.92 ± 0.12
Normal	Starved	0.551 ± 0.043		4.32 ± 0.97	
Starved	Normal	0.442 ± 0.034		10.90 ± 0.77	

Table 8. *Synthesis of ascorbic acid and of xylulose by mixtures of potassium chloride extracts from normal and starved rats*

Experimental conditions were as in Table 3, with 20 μ moles of potassium gulonate as substrate. Results are given as mean values $\pm$ S.E.M. of five experiments for each group.

Treatment	Ascorbic acid formed		Xylulose formed	
	(μ mole/200 mg. wet wt./hr.)	(μ mole/mg. of N/hr.)	(μ moles/200 mg. wet wt./hr.)	(μ moles/mg. of N/hr.)
Fed	0.649 $\pm$ 0.046	0.164 $\pm$ 0.014	3.96 $\pm$ 0.55	0.99 $\pm$ 0.13
Starved	0.468 $\pm$ 0.035	0.107 $\pm$ 0.006	10.93 $\pm$ 1.24	2.49 $\pm$ 0.24
50% Fed + 50% starved	0.585 $\pm$ 0.037	0.136 $\pm$ 0.011	4.78 $\pm$ 0.95	1.10 $\pm$ 0.21

synthesis was not restored to the level obtained with recombined fractions from normal rats by the addition of supernatant from normal rats to the sediment from starved rats.

Experiments with mixed extracts. The possibility of the presence of an activator or the removal of an inhibitor or both was tested to account for the changes observed in the synthesis of ascorbic acid and of xylulose. The usual procedure for detecting the presence of an activator or of an inhibitor is to assay the enzyme activity of control and experimental preparations individually and in combination. The amount of ascorbic acid formed by the mixture of extracts from normal and starved rats (Table 8) was almost exactly the average between the quantities formed by extracts from normal and starved rats incubated separately, so excluding the presence of an inhibitor. The amount of xylulose formed by mixtures of extracts from normal and starved rats was very close to that formed by extracts from normal rats alone.

DISCUSSION

Observations on the glucuronic acid pathways. According to Chatterjee *et al.* (1960a), Suzuki, Mano & Shimazono (1960) and Yamada *et al.* (1962) glucuronolactone could be converted into gulonolactone either by direct reduction or via gulonic acid after hydrolysis to glucuronic acid. The latter seems to be the prevalent pathway operating in the liver extracts used in present experiments. A first indication for this is the rapid disappearance of lactones. Since very little ascorbic acid is formed before lactones have disappeared (Fig. 1), transformation of glucuronolactone into gulonolactone or into ascorbic acid should be excluded at this point, and it should be assumed that glucuronolactone is hydrolysed to glucuronic acid unless it is metabolized through reactions outside those examined in the present work. Further, in normal rats more ascorbic acid is formed from glucuronate than from glucuronolactone, thus indicating that the former is probably a

closer precursor of ascorbic acid. The same amount of xylulose is formed from these two substrates, but this may not be in contrast with the view expressed above, since the conditions of incubation that are optimum for ascorbic acid synthesis might be not so for xylulose formation.

A lag phase has been noticed in the synthesis of ascorbic acid from glucuronolactone; since the end of this phase coincides approximately with the disappearance of the lactone, one could explain this as being due to the time required to transform glucuronolactone into glucuronate. This, however, must be excluded, since the same delay is observable with glucuronate as substrate.

Chatterjee, Ghosh, Ghosh & Guha (1958), Chatterjee *et al.* (1960a,b), Kar, Chatterjee, Ghosh & Guha (1962) and Suzuki *et al.* (1960) gave evidence for the direct conversion of glucuronolactone into gulonolactone and then into ascorbic acid by preparations of purified microsomes, provided that cyanide or pyrophosphate was added at relatively high concentrations. The present experiments with potassium chloride and sucrose extracts containing the supernatant as well as the microsomes and in which ascorbic acid is formed without the requirement for cyanide give probably a more physiological representation of what may occur in the whole living cell.

Direct transformation of glucuronolactone into gulonolactone is possible through the action of NADP-linked hexonate dehydrogenase; this enzyme, however, is much more active on glucuronic acid than on its lactone; further, the lactone is available only for a short time, owing to its hydrolysis by lactonase. Even if some gulonolactone were formed directly from glucuronolactone in our preparations, the activity of aldonolactonase is so high that most of it would be hydrolysed before being oxidized to ascorbic acid. Therefore gulonate should be considered the obligatory intermediate in ascorbic acid formation in the system used in present work. Since gulonate is also on the pathway leading to xylulose, it seems to be the 'key-stone' compound in the scheme of reactions considered, its

further metabolism depending on competition between the enzymes of each pathway.

Ishikawa & Noguchi (1957), Ishikawa (1959) and Chatterjee *et al.* (1960a) reported xylulose formation by a 'gulonic dehydrogenase' purified from the soluble supernatant and at that time supposed to be the only enzyme concerned. Smiley & Ashwell (1961) purified a 3-oxogulonate decarboxylase from guinea-pig-liver preparations containing presumably some particulate matter. The results of the present experiments with isolated and recombined fractions indicate that the presence of the sediment is essential for the transformation of gulonate into xylulose. Experiments with boiled fractions showed that the activity of both the sediment and the supernatant was destroyed by boiling, thus suggesting that each fraction contains an enzyme or an essential thermolabile enzymic cofactor. Since the soluble supernatant has gulonate-dehydrogenase activity, the requirement for the particulate fraction could be explained by one of the following possibilities: (i) the presence in this fraction of the 3-oxogulonate decarboxylase, or of an essential part or cofactor of this enzyme; this hypothesis is in contrast with the observations of the above-mentioned authors on the xylulose formation by supernatant preparations; (ii) the removal of an inhibitor; (iii) the need of this fraction for the operation of some different reaction(s) that could accelerate the dehydrogenation of gulonate by a coupled mechanism, as shown by Ishikawa & Noguchi (1957), Ishikawa (1959) and Isherwood *et al.* (1960) for the pyruvate-lactate dehydrogenase system. Also, the xylulose formed exceeds the amount of NAD present in the system, sometimes by a 3-4:1 ratio; therefore NADH must be reoxidized to allow the dehydrogenation of gulonate to proceed.

According to ul Hassan & Lehninger (1956) the ascorbic acid added to the system used for the synthesis is in part destroyed. It has been impossible to confirm this point, or to find out the reasons for this discrepancy. In the present experiments some destruction occurred only when NAD and ATP were omitted from the system; however, the elucidation of this point being beyond the purpose of this research, no experiments were performed to investigate which cofactor(s) was necessary to prevent destruction of ascorbic acid.

Effect of starvation. The impairment of ascorbic acid synthesis from glucuronolactone and glucuronate is far greater than that from gulonate or gulonolactone; xylulose formation from uronate precursors is also decreased during starvation. The impairment of microsomal gulonolactone oxidase can account for the altered conversion of gulonate into ascorbic acid, and indirect support is given by experiments with recombined fractions, which locate the alteration in the sedimentable fraction, and the

experiments with mixed extracts exclude the occurrence of an inhibitor of ascorbic acid synthesis in preparations from starved rats. The activity of the uronolactonase and aldolactonase and of the hexonate dehydrogenase is unchanged, thus indicating a normal capacity of formation of gulonate and gulonolactone from uronate precursors. Therefore the reason for the impaired formation of ascorbic acid and of xylulose from glucuronate and glucuronolactone is probably outside the enzymic reactions examined in the present work.

The increased formation of xylulose from gulonate and gulonolactone cannot be accounted for by the enhanced activity of the gulonate dehydrogenase, since this enzyme is present in the soluble supernatant (Chatterjee *et al.* 1960a; Isherwood *et al.* 1960; Smiley & Ashwell, 1961), whereas the cause of the alteration is in the sedimentable fraction, as shown by recombination experiments. The possibility of an enhanced activity of the 3-oxogulonate decarboxylase is also in contrast with the location of this enzyme in the soluble supernatant (Winkelman & Ashwell, 1961). Another possibility is that some coupled reaction(s) (Ishikawa & Noguchi, 1957; Ishikawa, 1959; Isherwood *et al.* 1960) may operate at an increased rate during starvation.

The experiments with mixed extracts from normal and starved rats indicated the presence in normal liver of an inhibitor of xylulose formation; this factor seems located in the sedimentable fraction. It remains to be ascertained whether the disappearance of this inhibitor is the only cause of the enhanced formation of xylulose during starvation or whether synthesis of new enzyme(s) is involved.

The hypothesis was formulated that the enhanced formation of xylulose could account for the impaired synthesis of ascorbic acid, from gulonate, since this intermediate could be competitively removed by the action of aldolactonase and gulonolactone oxidase. Since the synthesis of ascorbic acid is lowered approximately at the same extent even when formation of xylulose is suppressed by the omission of NAD, the changes in ascorbic acid and xylulose synthesis appear to be independent of each other, although the possibility remains that gulonate might be removed if it was bound by the gulonate dehydrogenase to form an enzyme-substrate complex, as postulated by Isherwood *et al.* (1960) for the synthesis of ascorbic acid.

Fitch & Chaikoff (1960), developing concepts introduced by Freedland & Harper (1959), postulated that '(a) the level of an enzyme's activity is related to the metabolic activity of the pathway in which the enzyme participates; (b) a change in this level reflects, qualitatively, alterations in usage (throughput) of that metabolic pathway'. The enhanced formation of xylulose from gulonate would suggest a preferential drift to the glucuronic acid

pathway during starvation, although formation of xylulose from glucuronate is decreased. Some indirect support for this hypothesis is given by the observations of Winegrad & Shaw (1964) indicating a stimulation of glucose metabolism through the glucuronic acid pathway in adipose tissue during starvation. However, some reactions of the pathways under study take place in the particulate fraction, and possible changes of permeability of subcellular organelles could be involved in the changes of enzymic activities observed during starvation. Further, it is not known exactly which enzyme is responsible for the increased formation of xylulose from gulonate; should it be gulonate dehydrogenase, which is a broadly unspecific enzyme, changes of its activity might be caused by metabolic alterations outside the glucuronic acid pathway. Whatever is the mechanism and the meaning of the changes observed, the fact that they are induced by starvation, carbohydrate deprivation and alloxan-diabetes (Stirpe & Comporti, 1963, and unpublished work) allows us to suppose that they are connected with alterations of carbohydrate metabolism.

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