3. The kinetics of the activation process were investigated.

4. The activated venom substrate has amino acid-esterase activity as well as a powerful coagulant activity.

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The Conversion of ^{14}C -Labelled 'Glucose Cycloacetoacetate' into L-Ascorbic Acid in Albino Rats

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The first isotopic investigation into the biosynthesis of ascorbic acid was that of Jackel, Mosbach, Burns & King (1950). They used uniformly (U) labelled $[\text{U-}^{14}\text{C}]$ glucose, which gave rise to $[\text{U-}^{14}\text{C}]$ -ascorbic acid in chloretone-stimulated rats. The work of Horowitz, Doerschuk & King (1952) and Horowitz & King (1953) supported the contention that in animals D-glucose is converted into L-ascorbic acid without fragmentation but with inversion of its carbon skeleton. A general scheme linking D-glucose and D-galactose with L-ascorbic acid was proposed by Isherwood, Chen & Mapson (1954), in which the postulated intermediates are:

This scheme is supported by work on animals, though not by the work of Loewus, Jang & Seegmiller (1956) and Loewus & Jang (1957) with strawberries and cress seedlings, in which glucose was converted into L-ascorbic acid without fragmentation, but, in contrast with rats, there was no inversion of the molecule. Galactose also passes through an intermediate triose pool, which symmetrically distributes the label between C-1 and C-6. This resembles the route taken by L-[6- ^{14}C]-

sorbose in chloretone-treated rats (Burns, Mosbach, Schulenberg & Reichenthal, 1955).

Work with *Phaseolus radiatus* by Nath, Chitale & Belavady (1952) also suggests that the scheme originally proposed by Isherwood *et al.* (1954) is not the only pathway leading to ascorbic acid formation from glucose. Although acetoacetate injection decreased the ascorbic acid content in animals (Nath, Sahu & Chitale, 1953*b*), it induced a slight increase when administered simultaneously with glucose (Nath, Belavady, Sahu & Chitale, 1953*a*). The condensation product of glucose and acetoacetate 'glucose cycloacetoacetate' gave a significant increase in ascorbic acid content of plasma of rats and germinated mung beans, and was postulated to be a precursor of ascorbic acid. The conversion of glucose cycloacetoacetate into ascorbic acid was confirmed by chromatography (Nath & Belkhode, 1959) and by biological studies with scorbutic guinea pigs (Nath & Belkhode, 1960). Radiochemical evidence indicated that $[\text{U-}^{14}\text{C}]$ glucose cycloacetoacetate gave rise to labelled ascorbic acid in germinating *Phaseolus radiatus* (Thangamani & Sarma, 1956). That glucose cycloacetoacetate is a constituent of normal blood has been reported by Nath, Behki & Sahu (1958). Work with $[\text{U-}^{14}\text{C}]$ - and $[\text{6-}^{14}\text{C}]$ -glucose has suggested that in the conversion of glucose cycloacetoacetate into L-

ascorbic acid in *Phaseolus radiatus* the carbon chain of glucose remains intact but is not inverted (Belkhode & Nath, 1962). Although the above findings are presumptive evidence for a pathway in plants in which glucose cycloacetoacetate is converted into L-ascorbic acid with maintenance of the intact carbon skeleton of the glucose, similar experiments have not so far been conducted with intact animals.

The present paper describes work with [$1\text{-}^{14}\text{C}$] and [$6\text{-}^{14}\text{C}$] glucose moieties of glucose cycloacetoacetate in chloretone-stimulated rats. It is known that some nerve depressants, such as chloretone and barbitone, stimulate rats to excessive urinary excretion of ascorbic acid (Musulin, Tuly, Longenecker & King, 1939; Longenecker, Musulin, Tuly & King, 1939; Longenecker, Fricke & King, 1940).

EXPERIMENTAL

Materials. D-[$1\text{-}^{14}\text{C}$]glucose and D-[$6\text{-}^{14}\text{C}$]glucose were purchased from The Radiochemical Centre, Amersham, Bucks. Amberlite IR-4B (OH^- form) was a product of Rohm and Hass Co., Pa., U.S.A.

Synthesis of [$1\text{-}^{14}\text{C}$]glucose cycloacetoacetate. The procedure suggested by West (1927) was used. D-[$1\text{-}^{14}\text{C}$]glucose (0.2 mc) was mixed with 20 g. of carrier D-glucose and 15 g. of ZnCl_2 in a glass mortar. The mixture was transferred to a round-bottom flask and, after the addition of 10 ml. of ethyl acetoacetate and 15 ml. of ethanol, it was heated for 30 min. in a boiling-water bath with continuous stirring. The syrupy mass was then poured into a beaker containing 50 ml. of water and kept in the refrigerator at 0° for crystallization. The mother liquor was separated by filtration and the crystals were washed with ice-cold water several times to remove adhering glucose. The product was recrystallized two to three times from hot water, and glistening white needles were obtained (m.p. $152\text{--}153^\circ$). The specific activity was 5.3×10^8 counts/min./mg. (windowless gas-flow counter, Nuclear-Chicago Corp.).

Synthesis of [$6\text{-}^{14}\text{C}$]glucose cycloacetoacetate. D-[$6\text{-}^{14}\text{C}$]glucose (0.2 mc) was mixed with carrier D-glucose and treated as described above. The specific activity of [$6\text{-}^{14}\text{C}$]glucose cycloacetoacetate was 4.1×10^8 counts/min./mg.

Recovery and isolation of ascorbic acid. Male albino rats weighing 100–120 g. were housed in metabolism cages in which all the metal parts in contact with urine were waxed. They were given daily doses of chloretone, increasing from 10 to 40 mg., through a stomach tube in 0.4 ml. of olive oil. When the ascorbic acid excretion in the urine reached 25–30 mg./day, as determined by 2,6-dichloroindophenol, about 1.5 ml. of a solution of [^{14}C]glucose cycloacetoacetate ($4\text{--}6\mu\text{C}$) was injected intraperitoneally. After 24 hr. the chloretone administration and [^{14}C]glucose cycloacetoacetate injection were repeated. Urine was collected during the 48 hr. period in 10 ml. of 10% (w/v) oxalic acid. The total ascorbic acid in the urine was determined and enough carrier was added to bring the total amount of ascorbic acid to 200 mg. The oxalic acid was removed by adding 1.9 g. of lead acetate for each 0.5 g. of oxalic acid added, and the urine was then passed through a column of

Amberlite IR-4B (OH^- form) (Jackel, Mosbach & King, 1951). The column was washed with 200 ml. of water and the ascorbic acid eluted with N-HCl. The solution was concentrated to dryness *in vacuo* at 40° and the crystalline residue was taken up in 5 ml. of ethanol, treated with ether and filtered. The filtrate was again concentrated to dryness *in vacuo* and the product recrystallized from acetic acid. The white crystals were washed with ether and dried (m.p. $190\text{--}191^\circ$).

Degradation of ascorbic acid. Ascorbic acid was degraded as suggested by Horowitz *et al.* (1952) and Horowitz & King (1953). C-1 was isolated as CO_2 by decarboxylation of ascorbic acid with 10 ml. of $8\text{N-H}_2\text{SO}_4$ under a stream of N_2 for 3 hr., and CO_2 was trapped in saturated $\text{Ba}(\text{OH})_2$ solution.

C-1 and C-2 were isolated together as oxalate by treating ascorbic acid with 15 ml. of 0.1N-iodine in 0.2N-KI followed by 5 ml. of N-NaOH. The solution was brought to pH 5.0 with 0.5 ml. of acetic acid and calcium oxalate precipitated by the addition of 1 ml. of M-calcium acetate.

C-6 was obtained from the osazone of ascorbic acid as suggested by Horowitz *et al.* (1952).

Radioactive purity and determination. A portion of ascorbic acid (15–20 mg.) recovered from urine was dissolved in 5 ml. of water and 1 ml. was applied to a weighed aluminium planchet. After drying on a steam bath, the planchet was again weighed, thus keeping the weight of ascorbic acid in each planchet between 3 and 4 mg. The radioactivity was counted and corrected for background counts only. The counts were converted into counts/min./m-mole. Carbon dioxide from the degradation of ascorbic acid was recovered as BaCO_3 , which was thoroughly washed with water and finally dried by washing with ethanol and ether. It was plated to minimum thinness and corrected for background and self-absorption and converted into counts/min./m-mole of ascorbic acid.

The osazone and dimedone-formaldehyde fractions of ascorbic acid were recrystallized to constant specific activity, the former from ethanol-acetone (1:1, v/v) and the latter from light petroleum (b.p. $100\text{--}120^\circ$). The activity of the osazone was corrected by using a BaCO_3 self-absorption curve (Horowitz *et al.* 1952), and the factor for the dimedone-formaldehyde fraction was calculated from a self-absorption curve obtained with uniformly labelled glucose by the method of Reeves (1941).

RESULTS

Table 1 shows the percentage distribution of ^{14}C in various fragments of ascorbic acid isolated from the urine of chloretone-treated rats, when [$1\text{-}^{14}\text{C}$]glucose cycloacetoacetate is administered intraperitoneally, and is based on the activities expressed in counts/min./m-mole of ascorbic acid. Of the activity 64–71% appears in C-6 and only 8–10% occurs in C-1. The ratio of activity between C-1 and C-6 shows that out of the total activity in both (C-1 + C-6) nearly 86–89% appears in C-6. The activity in C-2 appears to be less than in C-1, as found by difference between C1 + C-2 and C-1, which indicates that very few counts are contributed by C-2.

Table 1. *Percentage distribution of ^{14}C in various carbon atoms of ascorbic acid after administration of $[1-^{14}\text{C}]\text{glucose cycloacetoacetate}$*

Figures in parentheses represent the experimentally determined activity in counts/min./m-mole of ascorbic acid.

Expt. no.	Specific activity of ascorbic acid (counts/min./m-mole)	Percentage distribution of ^{14}C in each degraded fragment			^{14}C ratio [C-6/(C-1 + C-6)] $\times$ 100
		C-1	C-1 + C-2	C-6	
1	4160	8 (374)	12 (499)	71 (2956)	89
2	2038	10 (224)	14 (297)	64 (1324)	86
3	3325	9 (325)	14 (485)	65 (2194)	88

Table 2. *Percentage distribution of ^{14}C in various carbon atoms of ascorbic acid after the administration of $[6-^{14}\text{C}]\text{glucose cycloacetoacetate}$*

Figures in parentheses represent the experimentally determined activity in counts/min./m-mole of ascorbic acid.

Expt. no.	Specific activity of ascorbic acid (counts/min./m-mole)	Percentage distribution of ^{14}C in each degraded fragment			^{14}C ratio [C-1/(C-1 + C-6)] $\times$ 100
		C-1	C-1 + C-2	C-6	
4	2236	61 (1386)	66 (1498)	11 (246)	85
5	1785	64 (1145)	67 (1205)	8 (145)	89
6	2862	64 (1860)	68 (1974)	9 (258)	88

Table 2 records the percentage distribution of ^{14}C in various fragments of ascorbic acid, together with the experimentally determined counts/min./m-mole of ascorbic acid when $[6-^{14}\text{C}]\text{glucose cycloacetoacetate}$ is the labelled precursor. The distribution of ^{14}C suggests that C-1 is now predominantly labelled, i.e. 61–64 % appearing in C-1 and 8–11 % in C-6. The activity ratio between C-1 and C-6 shows that 85–89 % appears at C-1 of ascorbic acid.

DISCUSSION

The results presented show that glucose cycloacetoacetate labelled in C-1 and C-6 of the glucose, when administered intraperitoneally to chlorotone-treated rats, yields ascorbic acid predominantly labelled in C-6 or C-1 respectively. The yield of radioactive ascorbic acid was the same from either C-1- or C-6-labelled substrates. These experiments are in agreement with results from *Phaseolus radiatus* (Belkhole & Nath, 1962) showing that glucose cycloacetoacetate is converted into ascorbic acid with preservation of the intact carbon skeleton of the glucose; in the rat, inversion of the carbon chain occurs. The pattern of labelling we found resembles that observed by Horowitz *et al.* (1952) and Horowitz & King (1953) with D- $[1-^{14}\text{C}]\text{glucose}$ and D- $[6-^{14}\text{C}]\text{glucose}$ respectively in chlorotone-

treated rats. Results with $[^{14}\text{C}]\text{glucose cycloacetoacetate}$ (Belkhole & Nath, 1962) suggest that the radiochemical yield of ascorbic acid from $[^{14}\text{C}]\text{glucose cycloacetoacetate}$ in germinating *Phaseolus radiatus* was four times as great as that obtained with $[\text{U}-^{14}\text{C}]\text{glucose}$, indicating that glucose cycloacetoacetate is a better precursor of ascorbic acid than glucose. Similar observations were recorded by Thangamani & Sarma (1959) in rats.

These data support the findings of Nath *et al.* (1952) that the scheme proposed by Isherwood *et al.* (1954) is not the only route of ascorbic acid formation. Nath & Belkhole (1958) have reported the enzymic synthesis of glucose cycloacetoacetate when equimolar proportions of glucose and acetoacetate are taken up by germinating mung beans. Thangamani & Sarma (1959) have observed a similar synthesis from $[\text{U}-^{14}\text{C}]\text{glucose}$ and acetoacetate in rats. However, at present the nature of the enzyme(s) involved in the condensation of these two compounds is unknown. Thangamani & Sarma (1957) observed with albino rats that the injection of glucose cycloacetoacetate causes a significant rise in the urinary excretion of glucuronic acid; this was lowered in pantothenic acid deficiency, which also caused the excretion of unmetabolized glucose cycloacetoacetate, thus implicating pantothenic acid in the conversion of glucose cycloacetoacetate into glucuronic acid.

SUMMARY

1. [^{14}C]Glucose cycloacetoacetate was injected into chloretone-treated rats and the ascorbic acid isolated from the urine was degraded to determine the activities of carbon atoms 1, 2 and 6.

2. Intraperitoneal injection of [^{14}C]glucose cycloacetoacetate resulted in excretion of ascorbic acid with 64–71% of the activity in C-6 and 8–10% in C-1.

3. Ascorbic acid recovered from the urine of rats injected with [^{14}C]glucose cycloacetoacetate contained 66–68% of the activity in C-1 and 8–11% in C-6.

4. The results indicate that the glucose moiety of glucose cycloacetoacetate remains intact and simultaneously it undergoes inversion.

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Separation of Antigens by Immunological Specificity

1. METHOD FOR SEPARATING INDIVIDUAL ANTIGEN-ANTIBODY COMPLEXES FROM MIXED ANTIGENS AND ANTIBODIES

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This paper describes the initial stage of attempts to use serological specificity for the fractionation of protein and other antigens. The potential of such a method for resolving mixtures of antigens with similar chemical properties is indicated by the serological differentiation of bacterial strains and more recently by the results of immunological analysis of mixed antigens by gel diffusion and by immunoelectrophoresis. The success of such a method of fractionation would depend on three

main conditions. (a) A method should be found for isolating individual antigen-antibody complexes from the interaction of mixtures of unknown antigens with mixtures of corresponding antibodies. The authors are not aware of any previous method for obtaining monospecific complexes from such mixtures. Monospecific antibody has been prepared by immunochemical methods [see review by Isliker (1957)], but only when the appropriate single antigen was available either for preparing the