DISCUSSION

Previous studies from this laboratory showed that incubation of D-glucuronolactone with tissue homogenates from animals led to formation of a significant amount of L-ascorbic acid (46). It was, therefore, suggested that this *in vitro* technique might offer a simple means for determining the capacity of different species for the synthesis of ascorbic acid. However, the tissue homogenate contains lactonase, the enzyme which hydrolyzes D-glucuronolactone to the corresponding free acid (97). Since the ascorbic acid synthesizing enzyme is specific for the lactone form of the substrate and since D-glucuronic acid is inactive as a precursor, the lactonase obviously acts as an inhibitor to the biosynthetic mechanism (97). In the present investigation, therefore, microsomes along with homogenates were used as the source of enzyme. Since, L-gulonolactone is a more immediate precursor of ascorbic acid, this was also used along with D-glucuronolactone as the substrate. Different species of animals from the taxonomist's phylogenetic tree were investigated for their respective capacity to synthesize L-ascorbic acid.

While the invertibrates ended mainly with insects (Scheme II), probably the life of the vertibrate started with fishes (Scheme III). It was therefore, of interest to

investigate the biosynthetic capacity of both the insect and the fishes along with other species of animals.

Cockroach was selected as a typical insect not only because it is easily available but also due to the fact that it is a body with completely differentiated organs giving reproducible results throughout the investigation.

The cockroaches contained a significant amount of ascorbic acid. The content did not decrease even when the insects were maintained on ascorbic acid free pure cane sugar diet (Table I). This would indicate that ascorbic acid was formed in the body of the cockroach. Since ascorbic acid is synthesized either in the kidney tissue or in the liver, and since the fat body and the malphagian tubules of cockroach are analogous in some respect to the liver and kidney of mammals respectively, the homogenates from the fat body and malphagian tubules were examined for the biosynthetic capacity. However, none of the substrates examined, namely, mannose, glucose, fructose, galactose, xylose, sorbose, ribose, D-glucuronic acid, D-glucuronolactone, L-gulonolactone and L-galactonolactone were converted to ascorbic acid when incubated with homogenates from either fat body or malphagian tubules for a period upto three hours.

Assuming the enzyme concentration in the tissue from cockroach was small, the tissue concentration was increased

to two and half times, but no synthesis was obtained (Table IV). Neither, addition of fat body homogenate to an in vitro system containing goat liver microsomes caused any impairment of ascorbic acid biosynthesis (Table V). This would indicate that the fat body from cockroach did not contain any inhibitor of ascorbic acid synthesizing enzyme system.

Considering that the rate of biosynthesis in cockroach might be very small, the period of incubation was prolonged upto twentyfour hours. Though, no ascorbic acid synthesis was observed upto eighteen hours but the vitamin was formed when the incubation period was prolonged to twentyfour hours. This was contrary to the observation that the synthesis by goat liver homogenate was not increased over and above that obtained after three hours of incubation. Moreover, while in the case of goat liver D-glucuronolactone and L-gulonolactone acted as precursors, except mannose none of the sugars mentioned before was converted to ascorbic acid by the fat body. The ascorbic acid could not be estimated by titration with 2:6 dichlorophenol indophenol. It was estimated as 2:4 dinitrophenyl hydrazine derivative. Since, under the condition of incubation, the enzymic activity of goat liver tissue was completely lost after a period of six hours, the synthesis by the fat body was considered not to be carried out by enzymes. The fat body of cockroach contains intracellular symbionts and the

possibility existed that the observed ascorbic acid synthesis might be due to the microbial symbionts rather than to insect tissue enzymes. This expectation was confirmed by experiments using antibiotics. The ascorbic acid content of the whole body and the fat body as well as the biosynthetic capacity of the fat body from aposymbiotic cockroach were completely diminished. Also, the biosynthetic capacity by the fat body from normal cockroach was completely inhibited when chloramphenicol was added to the in vitro system. To rule out the possibility that chloramphenicol was inhibitory to the enzyme system concerned in biosynthesis, experiments were also done both in vivo and in vitro with rat. The urinary excretion, tissue storage and the biosynthetic capacity of liver were not diminished. Neither addition of chloramphenicol inhibited the in vitro synthesis by normal rat liver homogenate. Observation by Pierre (66) with fat body of Leucophae maderae led to similar conclusion that synthesis of ascorbic acid by the fat body was due to the symbionts and not to insect enzymes. Perhaps the same comment might be made on the synthesis of ascorbic acid by rice moth larvae (82). There is no evidence at present that the cockroach requires ascorbic acid or derives any advantage from the biosynthetic ability of its symbionts.

It has been reported that the fat body of silk worm pupae is able to effect synthesis of ascorbic acid from mannose (87). However, it has later been shown by growth studies using

synthetic diet that the silk worm requires ascorbic acid for satisfactory nutrition (88). This would indicate that mannose is probably converted to D-ascorbic acid in the fat body rather than L-ascorbic acid, the former having one-twentieth antiscorbutic activity. If the silk worm pupae synthesized L-ascorbic acid, it would not require dietary ascorbic acid for better nutrition. The formation of D-ascorbic acid from mannose is also supported by the observation of Mapson et al. (9), that injection of D-manno-γ-lactone in the rat led to the formation of D-ascorbic acid.

Estimation of L-ascorbic acid in different tissues of a number of fishes indicated that the hepatopancreas, the kidney and the brain contained a significant amount of L-ascorbic acid (Table IX). However, incubation of homogenates as well as different subcellular fractions including microsomes from kidney and hepatopancreas of various species of fishes with D-glucuronolactone and L-gulonolactone did not produce any ascorbic acid. Considering the possibility that the biosynthetic pathway of fishes were different from that of other animal system, but more inclined to that of the plant pathway, the tissues were also incubated with L-galactonolactone as substrate, but still no synthesis was observed. Since it was observed with birds (vide infra) that the highly evolved species could not synthesize ascorbic acid, fishes at different stages of evolution, including primitive and the

highly evolved forms, were examined, but no syntheses were observed in any case (Table X). Since fishes are cold blooded animals, the temperature of incubation was varied between 20-60°C but without any effect. Prolonging the incubation time, as done with fat body of cockroach, was of no value.

Neither the lactonase activity of fish tissue was significantly greater than that of rat tissue, nor the fish tissues contained any inhibitor of ascorbic acid synthesizing enzyme system. The results indicate that the capacity to synthesize L-ascorbic acid is absent in the fish. Or in otherwords, the biosynthetic capacity did not start in the primitive vertibrates.

The result, however, is at variance with that observed by the Japaneese workers (92) who obtained synthesis of ascorbic acid by carp hepatopancreas extracts and the synthesis was increased by addition of α-tocopherol in the incubation medium. Those workers (92) claimed that α-tocopherol inhibited lipid peroxidation in the fish tissue with the result that ascorbic acid synthesis was accelerated. Unlike rat liver microsomes, incubation of L-gulonolactone with shoule hepatopancreas microsomes did not give rise to formation of lipid peroxide (Table XVI). This would further indicate that no ascorbic acid was formed in the system, since addition of L-ascorbic acid produced lipid peroxide in the fish tissues. However,

this lipid peroxide, catalyzed by ascorbic acid, was inhibited by addition of sodium pyrophosphate or α-tocopherol in the incubation medium. But no synthesis of ascorbic acid took place even in the presence of sodium pyrophosphate and α-tocopherol (Tables X and XVII).

The ascorbic acid synthesis in fish tissue extract observed by the Japaneese workers was measured by decolorization of 2:6-dichlorophenol indophenol dye added in the incubation medium. Since dye decolorization takes place by non-specific reducing substances present in the tissue and since the Japaneese workers did not identify the biosynthesized ascorbic acid, it remained doubtful whether the value obtained by dye decolorization was due to ascorbic acid.

The biosynthetic capacity apparently started in the amphibia, the species evolved one step higher than the fish. The kidney tissue homogenate and the microsomes from the amphibia could convert both D-glucuronolactone and L-gulonolactone to L-ascorbic acid (Tables XX and XXI). This biosynthetic capacity of the amphibia is also located in the kidney of reptiles, the species having position higher to amphibia in the ascent of evolutionary order. Neither the liver nor the brain of these species contained the enzyme (Table XX).

When the more evolved species, namely, the mammals were examined, the enzyme system was found to be present in the

liver tissue and not in the kidney tissue (Table XX). When the more evolved primates were examined, namely, the man and the monkey, the biosynthetic capacity was altogether absent. Uptil now, it was known that the guinea pig and the primates were the only exceptions. However, when the two flying mammals, namely, the Indian fruit bat and the Indian pipistrelle were examined, neither the kidney nor the liver could effect the synthesis. The incapability of these flying mammals lends further biochemical evidence for the assumption that bat is closely related to primates.

Thus, in the evolutionary ascent, the capacity for synthesizing ascorbic acid, originally absent in the fish, started in the kidney of amphibia and reptiles, passed from the kidney to the liver of mammals and finally disappeared from the more evolved primates. This relationship between the biosynthetic capacity and species evolution appeared to be more pronounced when different species of birds at different stages of evolution in the phylogenetic scale were examined.

The results (Tables XXVI and XXVII) indicate that ... the birds which are placed in the older order of evolution of the phylogenetic scale, namely, the chicken, pigeon, koel, falcon, grey partridge, coot, common pochard, bareheaded goose, cattle egret, parakeet, owl and king fisher, synthesize ascorbic acid in the kidney and not in the liver. The biosynthetic capacity of the kidney is shared also by the

liver of two species in the passeriformes order, namely, the house crow and the common myna (Tables XXVI and XXVII). It would be interesting to note that in sharing the biosynthetic capacity by both liver and the kidney, the liver took the major share. It would appear that these two birds stand on the border line in passing the biosynthetic capacity from kidney to the liver. After house crow and common myna, the ascorbic acid synthesizing capacity has been completely taken over by the liver of bank myna, hill myna, pied myna, jungle myna, house sparrow, black headed munia, magpie-robin, tree-pie, orange headed ground thrush, wood pecker and jungle babbler, all of the passeriformes order. Mention should also be made of wood pecker, (Piciformes), whose position is almost parallel to the passeres (Scheme IV) and which synthesizes ascorbic acid in the liver and in the kidney. This is in agreement with the observation that birds in the higher order of evolution synthesize ascorbic acid in the liver and not in the kidney. The biosynthetic capacity by the jungle babbler is the smallest amongst the passeres mentioned and it appears to stand at the border line between the passeres those are ... capable of synthesizing ascorbic acid and those are not. Table XXVI shows that sixteen birds of the passeriformes order, placed after jungle babbler (Table XXVI) namely, swallow, sunbird; blackheaded oriole, flower pecker, paradise flycatcher, white spotted fantail flycatcher, Indian great

reed warbler, minivet, leaf bird, redvented bulbul, white cheeked bulbul, redwhiskered bulbul, white browed bulbul, grey shrike, baybacked shrike and rufousbacked shrike are incapable of synthesizing ascorbic acid. The result is at variance with the general assumption that practically all species except the guinea pig and the primates are able to synthesize ascorbic acid.

Attempt to identify 2,keto-L-gulonolactone, an intermediate in the conversion of L-gulonolactone to L-ascorbic acid was unsuccessful in the incubation medium containing tissue microsomes from 'Incapable' passeres. Neither those tissues could convert L-gulonolactone into L-ascorbic acid in absence of air using an artificial electron acceptor namely, phenazine methosulfate. This would indicate that the 'Incapable' birds lack L-gulonodehydrogenase.

Addition of tissue microsomes from 'Incapable' passeres did not inhibit synthesis of L-ascorbic acid by goat liver microsomes (Table XXX). This would eliminate the possibility of the presence of some inhibitors in the microsomes of 'Incapable' birds.

In order to investigate whether the enzyme in the kidney and that in the liver is similar in nature or not, some of the properties of the enzyme were studied. It would

appear that there is a little difference in the pH optima (Fig. 7) and the Michelies' constant (Fig. 4) of the enzymes from the kidney of pigeon and the liver of sparrow. However, this would not reflect any difference in the enzymic nature, since, in both the cases the enzymic activity was assayed in intact microsomes and not with pure enzymes.

The relationship between the capacity of synthesizing L-ascorbic acid and evolution of animals in the phylogenetic scale is strikingly similar to that between different species of birds and their branched evolution. In the animal system, the enzyme originally residing in the kidney of species of the older order passed on to the liver of more evolved mammals and finally disappeared from the most evolved primates. In the birds also the enzyme is located in the kidney of species placed in the older order. It then passed on to the liver of more evolved passeres and disappeared from sixteen birds of the same order. The number of 'Incapable' passeres is such that the loss of biosynthetic capacity cannot be attributed to be accidental. On the other hand, based on a single biochemical phenomenon of lack of biosynthetic capacity, it is hard to assume that the 'Incapable' passeres are more evolved than the capable ones.

As indicated in Scheme IV, the position of the birds in the ascent of evolution was defined by some technical

characters including the precise arrangements of the flexor and the extensor tendons of the four toes which, as in most birds, anatomically grasp the perch when the bird bends the ankle in settling down. Based on this classification, the swallow appears to be the least evolved among the passeres and it would be expected that the swallow should have synthesized ascorbic acid. However, neither the kidney, nor the liver of swallow could effect any synthesis. Evolution is a multi-phased process, acting at all levels in the protoplasmic hierarchy, and it produces at intervals organized systems whose survival is determined on the touchstone of adaptation. In the process of transformation from reptilian body into a compact flying machine, the birds had not only to survive over the ruling reptiles and the rising mammals but also to conquer the air and adapt themselves under tremendously varried conditions of nature. Thus envisaged, the loss of the biosynthetic capacity of the 'Incapable' passeres might be due to a result of adaptation. Biochemical differences in nerve endings in the red and white muscle fibers of the pectoralis muscles of different birds have also been reported to be a result of adaptation (98). In tracing ~~training~~ to the relation of hormonal methylation with phylogeny of birds, Dutta and Ghosh (99) observed a definite divergence from the proposed taxonomy in two species, namely, B. benghalensis (order - Piciformes) and H. smyrnensis (order - Coraciformes). However, the biochemical change between the 'Incapable' passeres and the capable ones

with regard to L-ascorbic acid synthesis may not be a simple example of mere divergence. The possibility of biochemical evolution of the 'Incapable' birds along the passériformes branch of taxonomist's phylogenetic tree is not ruled out.

It is apparent from Scheme I that the synthesis of ascorbic acid is competitive with L-xylulose synthesis. The possibility, therefore, existed that the species which were incapable of synthesizing ascorbic acid would synthesize more xylulose than those capable of synthesizing the vitamin. Contrary to the expectation it would appear from Table XVIII that xylulose synthesis in different species of fishes is very small. Of course, whether the glucuronic acid pathway of glucose metabolism via the formation of xylulose exists in fish tissue at all or not is yet to be determined. While the synthesis of L-xylulose by some 'Incapable' passeres, namely, the bulbuls, is very high in comparison with that by pigeon and sparrow, the synthesis by shrikes is much less than that by other birds capable of synthesizing L-ascorbic acid (Table XXXII).

It should be mentioned, while the synthesis of ascorbic acid takes place in the microsomes, the synthesis of xylulose takes place in the soluble supernatant. Irrespective of the positions of the species in the phylogenetic scale and irrespective of whether the site of synthesis of ascorbic

acid is the liver or the kidney or whether the species is altogether capable or not of synthesizing the vitamin, both the kidney and the liver synthesized L-xylulose, the synthesis in the kidney was always greater than that in the liver (Tables XXIII and XXXII).

As has been mentioned before, irrespective of the species concerned, the enzyme system converting D-glucuronolactone and L-gulonolactone to L-ascorbic acid is located in the microsomes. While no added factor was necessary for the conversion of L-gulonolactone, a relatively high concentration of potassium cyanide was needed for the conversion of D-glucuronolactone into L-ascorbic acid. Until recently, cyanide could not be replaced ~~by cyanide could not be replaced~~ by any other metabolic inhibitors or reducing agents.

Recent experiment with rat liver microsomes (96) indicates that cyanide is not specific for this conversion and can be replaced by other aldehyde agents, namely, semicarbazide and hydroxylamine. In the present investigation also, it has been shown (Table XXXIII) that irrespective of whether the enzyme is present in the kidney or the liver, the microsomes from all the species examined can effect conversion of D-glucuronolactone to L-ascorbic acid in presence of semicarbazide and hydroxylamine. Table XXXIII further shows that the species which are incapable of synthesizing the vitamin in presence of cyanide are also incapable of doing so when cyanide is replaced by.

semicarbazide and hydroxylamine. The mechanism of action of aldehyde agents was traced to the fact that a freshly prepared solution of D-glucuronolactone contains in equilibrium a significant amount of the free aldehydo form which is inhibitory to the synthesis. Unless the aldehydo form is trapped by addition of an aldehyde agent, D-glucuronolactone is not converted into L-ascorbic acid. This is supported by the fact that conversion of D-glucuronolactone was inhibited non-specifically by a number of aldehydes, and the inhibition was reversed by a corresponding increase in the concentration of the aldehyde agent (Table XXXIV).
