VITAMIN C CONTENT OF MONKEY TISSUES IN EXPERIMENTAL POLIOMYELITIS*

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During the past few years much attention has been given to the study of vitamin C metabolism in health and disease. What has rendered this problem so attractive to laboratory workers and clinicians alike is the fact that vitamin C is not only significant as an accessory factor in nutrition, absence of which causes characteristic degenerative lesions in various tissues, but that ascorbic acid has come to be recognized as a physiological substance of great biochemical potency which seems to play an important part in the regulation of certain cell functions concerned with the mechanism of normal defense against microbial injury (1). This is borne out by numerous observations on increased susceptibility to bacterial infections and intoxications in C-deficient animals and proven more directly by the discovery that the vitamin C stores are often severely depleted during the progress of an infectious process. The latter data are based on vitamin C assays of the tissues of experimentally infected animals, as well as on the demonstration of C deficiencies in patients suffering from infectious diseases as determined by the urinary excretion following oral administration of large doses of ascorbic acid.

As might be expected, attempts have been made to compensate for this loss of vitamin C by increasing the intake; and evidence is beginning to accumulate which suggests that the administration of vitamin C during the course of certain infections and intoxications in man and animals may be followed by a reduction in severity of the pathological process. It is an open question whether such resistance-enhancing effects are due to an indirect stimulation of the physiological defense

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mechanism of the cells or whether ascorbic acid is capable of destroying directly certain bacteria, toxins, and viruses *in vivo*, as has clearly been demonstrated to occur when such infectious agents are brought together with the vitamin *in vitro*. While changes in vitamin C levels, occurring as they do in the wake of many infectious processes, do not necessarily signify any causal relationship to susceptibility, it has seemed possible that vitamin C metabolism may assume a particular significance in those infectious diseases which are normally characterized by wide variations in individual resistance. This would seem to apply especially if such infections or intoxications should prove to respond favorably to therapeutic doses of ascorbic acid as appears to be true for diphtheria, tuberculosis, and poliomyelitis (2), to quote only the best studied examples. It is for this reason that we have undertaken a detailed investigation of the vitamin C content of monkey tissues in experimental poliomyelitis, the object of this study being to compare the vitamin C levels of various representative tissues in normal monkeys with those of poliomyelitic monkeys and of infected animals in which an attempt had been made to lessen the severity of the infection by parenteral administration of vitamin C during the incubation period of the disease (2).

Methods

The work was planned to provide as fair a basis of comparison as possible between the vitamin C levels of normal and poliomyelitic monkeys which had received no injections of ascorbic acid and those of normal and infected animals which had been injected daily for 2 weeks with doses of ascorbic acid ranging from 5 to 100 mg. All animals were kept on the same general balanced dietary régime, which consisted of rice, bread, and milk and included an ample amount of bananas, oranges, and lettuce as a source of vitamin C. While the quality of the diet was therefore roughly adequate, no strict control of the quantitative individual intake of food could be obtained since the monkeys were sitting in groups of 10 to 15 in large cages. All monkeys were of approximately similar weight, ranging from 2300 to 2800 gm., and were sexually immature. The tissues studied were particularly brain, cord, and suprarenal; but we have also run numerous titrations of other organs such as liver, spleen, gonads, kidney, and intestines. Few tests were done with blood and spinal fluid since it was soon discovered that these fluids contained none or so little vitamin C that the amounts could not be accurately measured. Normal monkeys were sacrificed from time to time together with poliomyelitic monkeys so as to control any variables that might result from

the fact that this study extended over a period of several months (January to May) and that the animals were received from different shipments. Poliomyelitic monkeys, if prostrated, were killed at the height of paralysis in order to exclude any gross error due to inanition.

The monkeys (*Macacus rhesus*) were killed by intracardiac injection of air, and the tissues immediately removed and titrated for vitamin C according to the modified method of Bessey and King (3). This method measures only the reduced vitamin C present in the tissues, and is based on the fact that vitamin C, in strongly acidic solutions (of pH 2–3), will reduce very rapidly the indophenol reagent to its colorless base. The dye is blue in alkaline and pink in acid solution; the end-point of the titration is reached when the acid extract turns a pink color. The tissues are weighed accurately and then ground with sand in a mixture of 8 per cent trichloracetic and 2 per cent metaphosphoric acid in a glass mortar until a very fine homogeneous suspension is obtained. This suspension is centrifuged and the supernatant poured into a volumetric flask. The residue is again extracted with acid and centrifuged; the supernatants are then brought up to a volume of 25 or 50 cc., depending on the expected concentration of C in the tissues. Aliquot portions of the extract are titrated against sodium 2-6 dichlorobenzenone indophenol (Eastman Kodak P 3463). The dye is usually made up so that 1 cc. of the solution is the equivalent of 0.1 to 0.3 mg. of vitamin C. The indicator is standardized against an acid solution of crystalline vitamin C, which in turn has been standardized against a 0.01 N solution of iodine. The titrations are completed within about 30 seconds and the first pink color that appears is taken as the end-point. A blank of the acid mixture alone is always run for any given volume of extract. Other substances in tissue extracts will reduce the indicator even in strongly acid solution, although more slowly than vitamin C (4). For this reason a rapid end-point (5 to 30 seconds) is considered fairly accurate; however difficulties are often encountered when tissue extracts (*e.g.*, liver, kidney) contain substances which reduce the dye almost as rapidly as does vitamin C. The end-point is such that although it is rapidly fading, another drop of indicator is not immediately reduced by the extract as it would be if vitamin C were still present. The results, with correction for the blank, are calculated as mg. of vitamin C per gm. of tissue. The experimental error lies between 5 and 10 per cent. While the use of the indophenol reagent, therefore, may not give absolute values for the C content of tissues, we agree with others that it is accurate enough for comparative purposes, especially since the titration values against the dye are found to correlate closely with the values obtained by the biological assay method (5).[1]

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[1] Reversibly oxidized vitamin C may be brought back to the reduced state by the passage of H_2S through the solution for a length of time with subsequent removal of the H_2S by N or CO_2. Since substances other than dehydroascorbic acid may be reduced by H_2S and included in the titration, and since the routine

RESULTS

A total of 123 monkeys was killed for tissue titration. This number is made up of four main groups which are listed as follows: (1) normal monkeys which had not received any vitamin C parenterally, (2) normal monkeys which had been injected subcutaneously with 5 or 25 mg. of ascorbic acid daily for a period of 2 weeks, (3) control monkeys which had not received vitamin C and showed various degrees of paralysis following intracerebral inoculation with poliomyelitis virus, and (4) vitamin C-treated monkeys which had received subcutaneous injections of 5 to 100 mg. of ascorbic acid daily for a period of 2 weeks following intracerebral infection with poliomyelitis virus. This last group is further subdivided into monkeys which survived without paralysis following C treatment and those which succumbed to the disease in spite of treatment. The results obtained in the several groups of monkeys will now be discussed individually.

Group 1.—Normal monkeys. There were 5 normal monkeys in this group. These were sacrificed from time to time during the course of this investigation after being kept on the ordinary feeding schedule for a period of at least 2 weeks. The average C levels found in the central nervous system and suprarenals, expressed in mg. per gm. of tissue, were as follows: 0.08 for brain, 0.07 for cord, and 0.43 for suprarenal.

Group 2.—Normal C-prepared monkeys. This group included 3 monkeys all of which were sacrificed on the day following the last injection of vitamin C. The average C levels found in nervous tissue and the suprarenals of these animals were markedly higher than those observed for normal monkeys, *i.e.*, 0.15 for brain, 0.10 for cord, and 0.75 for suprarenal.

Group 3.—Poliomyelitic control monkeys. This group comprised a total of 24 animals serving as untreated controls for vitamin C-treated monkeys which had been infected simultaneously. Of these 24 animals 11 were completely prostrated by quadriplegia; the remaining

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use of this method was not practical we have run only a few such tests. In those instances where this method was used we found no changes in our values that could not be accounted for by probable errors in technique. It seems, therefore, likely that most of the vitamin was present in these tissues in the reduced form.

13 were partially paralyzed to various extents. The 11 completely paralyzed animals were killed at the height of paralysis and the 13 partially paralyzed animals at different stages of their convalescence, which varied from 3 to 4 weeks (7 animals) to 6 to 10 weeks (6 animals) following the onset of paralysis. The C levels found in animals killed at the height of paralysis as well as in the early stage of convalescence were uniformly low, average values of 0.07 and 0.06 being obtained for brain, 0.05 and 0.07 for cord, and 0.27 for suprarenal. These figures were slightly below those of corresponding normal monkey tissues. Somewhat higher average figures were observed in animals which were allowed to recover up to 10 weeks, *i.e.*, 0.13 for brain, 0.10 for cord, and 0.53 for suprarenal. It will be noted that these values were slightly above the C levels of normal monkeys.

Group 4. C-treated infected monkeys. This group is by far the largest, including a total of 91 monkeys in which an attempt had been made to influence the course of experimental poliomyelitis by parenteral administration of vitamin C during the incubation period. The total number of animals in this group is divided into two subgroups, the first subgroup representing 58 animals which developed paralysis in spite of treatment, while the second subgroup contained 33 animals which had survived without showing any evidence of paralytic symptoms.

The paralyzed animals, if prostrated by quadriplegia, were killed at the height of paralysis (35 animals) and if partially paralyzed were sacrificed at 3 to 4 weeks (19 animals) or at 5 to 10 weeks (4 animals) following the onset of paralysis. Titration of the tissues of these C-treated paralyzed animals showed average C values distinctly higher than those observed for untreated poliomyelitic controls, running from 0.13 to 0.14 for brain, from 0.09 to 0.11 for cord, and from 0.51 to 0.79 for suprarenal. These values were therefore either very slightly below or practically identical with the levels of normal C-prepared monkeys. There was little if any secondary rise in the figures concomitant with the progress of convalescence in this group, probably because the initial values were already high.

Interesting results were obtained in the second subgroup with C-treated animals which had escaped paralysis. These animals were sacrificed either between the 3rd and 4th week of survival following

the day of infection (21 animals) or as late as 6 weeks thereafter (12 animals). The average figures for those killed at the earlier period showed unusually high C levels in all tissues examined, *i.e.*, 0.16 for brain, 0.13 for cord, and 0.90 for suprarenal. However, when such animals had been permitted to live until 6 weeks after infection, these hypernormal values had again returned to figures which were only slightly above the normal average, *i.e.*, 0.13 in brain, 0.09 in cord, and 0.44 in suprarenal.

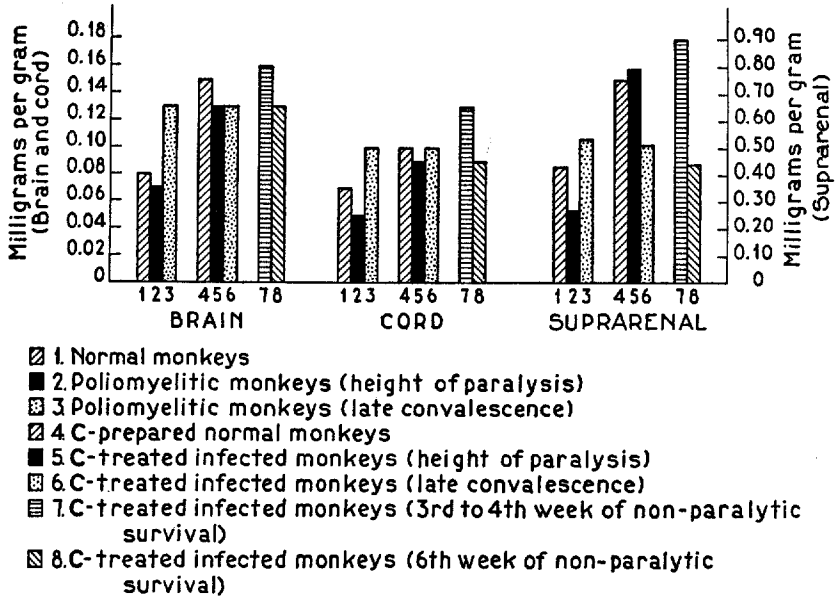

CHART 1. Vitamin C content of monkey tissues in experimental poliomyelitis.

A summary of these titrations will be found in Table I, while Chart 1 gives a comparison of the C levels, tissue for tissue, in the different groups of animals.

As was mentioned before, we have also run numerous C titrations on tissues other than the central nervous system and the suprarenals. These determinations have yielded results, closely comparable to those reported above, *i.e.*, high or low levels of C in the brain, cord, and the suprarenals were usually reflected by high or low levels in the spleen, liver, gonads, and intestines. These data are therefore not reproduced

TABLE I

Vitamin C Content in the Brain, Cord, and Suprarenal of Normal and Poliomyelitic Monkeys (with and without Parenteral C-Administration)

Group	Type of animal	Number of animals	Brain		Cord		Suprarenal	
			Median range per gm.	Average per gm.	Median range per gm.	Average per gm.	Median range per gm.	Average per gm.
			mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	Normal monkeys	5	0.04–0.11	0.08	0.05–0.10	0.07	0.25–0.69	0.43
2	Normal monkeys, C-prepared	3	0.15	0.15	0.09–0.10	0.10	0.66–0.86	0.75
3	Poliomyelitic control monkeys							
	Killed at height of paralysis	11	0.05–0.10	0.07	0.04–0.07	0.05	0.11–0.59	0.27
	Killed during early convalescence	7	0.04–0.09	0.06	0.05–0.10	0.07	0.09–0.40	0.27
	Killed during late convalescence	6	0.07–0.15	0.13	0.08–0.12	0.10	0.19–0.65	0.53
4	C-treated infected monkeys							
Subgroup 1	Killed at height of paralysis	35	0.08–0.16	0.13	0.08–0.11	0.09	0.52–1.04	0.79
	Killed during early convalescence	19	0.11–0.16	0.14	0.09–0.16	0.11	0.35–0.78	0.61
	Killed during late convalescence	4	0.09–0.17	0.13	0.07–0.12	0.10	0.18–0.83	0.51
Subgroup 2	Killed during 3rd to 4th wk. of non-paralytic survival	21	0.12–0.22	0.16	0.10–0.16	0.13	0.54–1.25	0.90
	Killed at 6th wk. of non-paralytic survival	12	0.11–0.16	0.13	0.08–0.11	0.09	0.24–0.86	0.44

in detail. However, we are giving in Table II and in Chart 2 the results of the titration of all tissues examined for normal monkeys and

TABLE II

Vitamin C Content of the Tissues of Normal and Vitamin C-Prepared Monkeys

Type of animal	Suprarenal per gm.	Gonad per gm.	Spleen per gm.	Liver per gm.	Brain per gm.	Cord per gm.	Intestine per gm.	Kidney per gm.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Normal	0.57	0.19♂	0.21	0.06	0.08	0.05	0.09	0.02
"	0.25	0.50♂	0.11	0.10	0.09	0.06	0.04	0.02
"	0.69	—	0.32	0.10	0.11	0.10	0.06	0.03
"	0.27	0.23♂	0.14	0.05	0.07	0.09	0.07	0.02
Normal C-prepared (5 mg.)	0.66	0.53♂	0.41	0.16	0.15	0.10	0.21	0.05
Normal C-prepared (25 mg.)	0.75	0.64♀	0.47	0.27	0.15	0.10	0.15	0.07
" " " " "	0.86	0.25♀	0.31	0.17	0.15	0.09	0.18	0.07

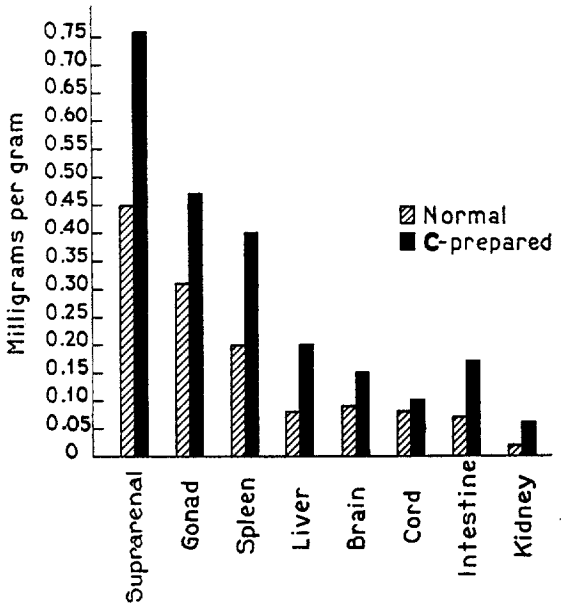

CHART 2. Vitamin C content of the tissues of normal and vitamin C-prepared monkeys.

normal C-prepared monkeys because they demonstrate clearly the increase in all values following preparation with vitamin C. These

increases are most marked in the liver, kidney, and intestine, and are followed by those in the spleen and brain. The cord seems to participate only to a very modest extent in this process of C assimilation.

DISCUSSION

The data presented in this paper offer information on the concentrations of vitamin C that are present in various tissues of normal *rhesus* monkeys and the extent to which such concentrations are subject to alteration by the parenteral administration of ascorbic acid. They are also helpful in explaining the results obtained with vitamin C therapy in experimental poliomyelitis.

It is well known that different animal species, on the basis of their susceptibility to experimental scurvy, may be divided into those which are capable of synthesizing their own vitamin C and those which depend upon dietary intake to supply this nutritional factor. There is well documented evidence to show that species of the first category, such as rats, rabbits, dogs, cats, and cattle, carry higher C levels in all tissues than man and the guinea pig, which belong to the second category (6). Although the monkey's susceptibility to experimental scurvy is well established, figures relating to the C content of monkey tissues, with the exception of the blood (7), have been wanting. The results of our titrations show that the C levels of the monkey fall in line with those of other non-synthesizing species. If the brain or suprarenal are taken as representative organs, the following series of C values, expressed in mg. per gm. of tissue, is therefore obtained: rat: 0.30 mg. (brain), 3 to 6 mg. (suprarenal); rabbit: 0.20 mg. (brain), 2 to 3 mg. (suprarenal); cat: 0.20 mg. (brain), 1 mg. (suprarenal); dog: 0.19 mg. (brain), 0.9 mg. (suprarenal); guinea pig: 0.14 mg. (brain), 0.3 to 0.7 mg. (suprarenal); man: 0.15 mg. (brain), 0.5 mg. (suprarenal); and monkey: 0.08 mg. (brain), 0.4 mg. (suprarenal). The values are slightly higher in very young and slightly lower in very old animals. Similar ratios are found in other tissues.

Since the amount of vitamin C found in the tissues depends upon the amount of ascorbic acid taken in, both orally and parenterally, C titrations must always be interpreted in terms of dietary intake. How sensitive an indicator the tissue C level is of variations in diet, other factors remaining constant, was forcibly brought to our attention in

this work. For a short time some monkeys were fed more oranges than usual, with the result that the C values ran significantly higher. On the other hand, some monkeys which were sacrificed shortly after having been received from the dealer, showed considerably lower C levels in all tissues. These animals were therefore excluded from tabulation in this study. We have also found some evidence for the presence of a slight seasonal increase in tissue C levels of all monkeys, normal, treated, and controls, beginning with the month of June. For this reason we have omitted all titrations carried out after that date. Even when due allowance has been made for variables which might affect the titration values, there still remains an extraordinary variability in the figures for animals within a given group, particularly in the suprarenal, as indicated by the extent of the so called median range from which the averages were computed.

The data obtained in this study have an important bearing on the nature of the therapeutic effect of vitamin C in experimental poliomyelitis. There is some indication that the amounts of C present in nervous tissues and in the suprarenal tend to diminish slightly during the progress of the infection. This is shown more clearly by contrasting the C levels of normal monkeys with those of poliomyelitic controls at the height of paralysis or during early convalescence than by a comparison of the figures obtained in normal and paralyzed animals which carried an additional load of vitamin C. As far as the suprarenal is concerned, this loss of C during the disease is in accord with scattered preliminary observations previously reported (8). However, it appears that the loss of C that occurs during poliomyelitic infection in the monkey is not nearly as striking as the C depletion observed in diphtheritic intoxication (9). In favor of the assumption that the lower C levels in paralytic monkeys are due to a temporary derangement of C metabolism produced by the disease and not referable to chance factors is the fact that poliomyelitic monkeys show a distinct tendency for a return to normal and even slightly hypernormal C levels as convalescence progresses. It is difficult, of course, to exclude with certainty that this secondary increase may not be the result of better adjustment to laboratory conditions. However, the data recently published by Torrance (10), who observed a marked increase in C content of the suprarenal in guinea pigs following

administration of sublethal doses of diphtheria toxin, suggest at least the possibility that recovery from poliomyelitis may be accompanied with a mobilization of vitamin C. Of particular interest is the fact that unusually high C levels were uniformly encountered in monkeys which had escaped paralysis following treatment with vitamin C, when such animals were examined in the early stages of their survival. Since these values were even higher than those obtained in normal animals after C preparation, they probably represent the aggregate effect of increased intake and increased assimilation of vitamin C during the infection. The artificial character of this high C saturation is clearly indicated by its brief duration, there being a return to normal levels in non-paralytic survivors which had not been examined until 6 weeks following the date of infection. Comparable figures have only been found in a small number of monkeys, not included in this work, which had passed through a non-paralytic febrile episode following intracerebral injection of poliomyelitis virus in combination with certain inactivating agents. It would seem likely therefore that the therapeutic effect observed after parenteral administration of vitamin C consists essentially in changing the frank paralytic type of the disease into the non-paralytic abortive type which fails to induce specific immunity to reinfection (11).

In conclusion it can be stated that distinctly hypernormal C levels in the central nervous system and in the suprarenals have been observed only in association with resistance to the disease. The possibility suggests itself, therefore, that there may be a certain delimiting threshold concentration of vitamin C in the tissues above which the monkey is protected and below which it is unprotected against the production of nerve cell damages by the virus. As this concentration begins to drop following discontinuance of C administration there is evidently a return to the state of susceptibility as indicated by the considerable number of animals which develop delayed paralysis between 14 days and 47 days following infection (12). We have no explanation to offer why only a certain percentage of infected monkeys following C administration are capable of reaching a critical concentration of vitamin C in the tissues and succeed in escaping paralysis permanently. But our data suggest that the success or failure of C treatment in experimental poliomyelitis may be intimately

connected with the extent to which the ascorbic acid is utilized by the organism.

SUMMARY AND CONCLUSIONS

1. The concentrations of reduced ascorbic acid present in the tissues of normal *rhesus* monkeys are of a magnitude in keeping with the values found for other animal species which are incapable of synthesizing vitamin C. These concentrations are subject to distinct increase by prolonged parenteral administration of ascorbic acid.

2. The amounts of vitamin C present in nervous tissue and the suprarenals of monkeys, paralyzed as the result of poliomyelitis infection, are slightly below the normal average when examined at the height of paralysis or in early convalescence. The figures show a tendency for a return to normal or slightly hypernormal levels concomitant with the progress of convalescence.

3. Vitamin C titrations of the tissues of monkeys which had received parenteral injections of ascorbic acid during the incubation period of poliomyelitic infection give different results according to whether such animals develop paralysis or survive without paralytic symptoms. In paralyzed C-treated monkeys the vitamin C levels are practically identical with those of normal C-prepared monkeys. Markedly higher values, however, are obtained with non-paralytic survivors in the early stages of their survival. As the period of survival lengthens normal figures prevail again.

4. The data are discussed in their relationship to the success or failure of vitamin C therapy in experimental poliomyelitis.

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