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STUDIES OF THE PROLONGED BIOCHEMICAL EFFECTS OF 3-METHYLCHOLANTHRENE AND OF ITS PHYSIOLOGICAL DISPOSITION IN THE RAT*

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Abstract—The intraperitoneal or subcutaneous injection of rats with 3-methylcholanthrene resulted in increased ascorbic acid excretion and enhanced activity of zoxazolamine hydroxylase for at least 7 weeks. The long-lasting biochemical effects of the hydrocarbon can be explained by its presence in the animal for a prolonged period of time.

In contrast to barbital, both Chloretone and 3-methylcholanthrene caused only a slight increase in free D-glucuronic acid excretion; all three compounds markedly stimulate L-ascorbic acid excretion.

It was found that in the initial 24 hr following administration, Chloretone produced a marked increase in ascorbic acid excretion and a moderate rise of zoxazolamine hydroxylase activity. Under similar conditions in the initial 24-hr period, 3-methylcholanthrene caused no appreciable effect on ascorbic acid excretion but led to a marked effect on the enzyme. Although both effects eventually paralleled each other, the fact that their onset was not simultaneous suggests that these two biochemical responses may not be interrelated.

SOME structurally unrelated compounds such as barbital, Chloretone (trichloro-*tert*-butyl alcohol) and 3-methylcholanthrene (MC) increase the biosynthesis, metabolism, and excretion of L-ascorbic acid in the rat.[2, 3] In addition, these compounds cause increased activity of drug-metabolizing enzymes in liver microsomes.[2] MC differs from drugs such as barbital and Chloretone in that its effects on L-ascorbic acid biosynthesis persist over a considerably longer period of time. The present study was designed to investigate further the effect of MC on drug-metabolizing enzymes and to determine whether the physiological disposition of MC could account for its prolonged biochemical actions.

METHODS AND MATERIAL

Animals and urine collection. Male Wistar rats (180–340 g), Twin Oaks Farms, Moorestown, N. J., were maintained on a 1:1 mixture of water and evaporated milk

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(Carnation) for one week prior to and during the experiments; this diet is low in L-ascorbic acid.

MC (Eastman-Kodak) was administered as a 2% w/v solution in corn oil (Mazola) either via interaperitoneal or subcutaneous (interscapular region) injection or by stomach tube. Chloretone was administered intraperitoneally in corn oil or added to the milk diet.

24-hr urines were collected from a metabolism cage placed over a 10-in. polyethylene funnel equipped with an iron mesh screen. For L-ascorbic acid measurements, urines were preserved in the presence of 5 ml of 8% oxalic acid; for free D-glucuronic acid analyses, collections were made with 1 ml of toluene.

Measurement of L-*ascorbic acid and free* D-*glucuronic acid.* L-Ascorbic acid in urine was determined by the indophenol dye method,[4] and free D-glucuronic acid was measured by a previously described method,[5] except that 3 ml of ethanol was used in the extraction procedure. This modification allows a more complete extraction of the colored complex from the formic acid solution, leading to increased recoveries; i.e. from 85 per cent to 95–100 per cent. The naphthoresorcinol used in the method for assay of D-glucuronic acid was purchased from Schwarz Bioresearch, Inc., Mt. Vernon, N.Y.

Measurement of 3-methylcholanthrene. MC was determined by a modification of the method of Dao *et al.*[6] Thiophene-free benzene (Mallinckrodt, Fisher, or Baker) was purified by shaking for 10 min with 10g/l. of activated charcoal (Merck or Penick) followed by two or more filtrations to remove all traces of charcoal. Benzene prepared by this procedure was found to be equivalent in 'blank' to fluorometric grade benzene (Harleco).

Because of the low concentration of MC in certain samples, special precautions were taken regarding the use of clean glassware and instruments. Glassware for MC determinations was cleaned successively with detergent, dichromate–sulfuric acid solution, ethanol, and benzene. Surgical instruments were treated in the same manner, except that they were not cleaned with dichromate solution. As in the method of Dao, tissues were refluxed with alkaline ethanol for a total of 6–8 hr. The alkaline solutions were extracted three times with 5 ml benzene/g tissue, and the mixtures were centrifuged (200*g*) for 10 min to separate the phases. MC concentrations were determined with an Aminco–Bowman spectrophotofluorometer calibrated against the Hg 546 mμ line. The instrument was checked for reproducibility with a Zeiss glass standard, obtained from Brinkman Instrument Co., Great Neck, N.Y., and polished on all four sides (Fish-Schurman, New Rochelle, N.Y.). The activation wavelength for this standard was 395 mμ and it fluoresced at 530 mμ. Maximal sensitivity and an IP 21 phototube was used for all measurements.

MC dissolved in benzene has major activation peaks at 295 and 362 mμ and major fluorescence peaks for each activation* at 400 and 420 mμ[6]. These maxima were used for MC analyses in all tissues except liver and spleen. For these two tissues, because of a shift due to complex formation with unknown factors, the activating wavelength employed was either 380 or 289 mμ, and the fluorescent wavelength was 400 mμ. The final choice of optimal wavelength depended on the 'blank' properties of the

* Chaudet and Kaye[7] reported that 3-methylcholanthrene in hexane has similar activation and fluorescence peaks.

various samples. Based upon the concentrations in the benzene extracts, either slit setting 2 or 3 was utilized.

Amounts of MC comparable to those found in experimental tissues (2–850 μg) were generally added to 10 g of normal tissues in order to determine recoveries and were analysed simultaneously with the samples under study. With most tissues, recoveries ranged from 80–100 per cent. In the case of liver and spleen, recoveries ranged from 100–120 per cent, apparently because of complex formation between MC and tissue constituents. Specificity for the analysis of MC was checked by the method of Dao,[6] using chromatography of the benzene extracts of tissues with silica gel columns (mesh: 100–200, Davison Chemical Co.).

In animals injected subcutaneously with MC and later skinned, fluorescence could be detected readily in the subdermal tissues. Therefore, analyses for MC at the injection site were made only on tissue samples from the dorsal areas, inclusive of skin, which showed fluorescence (8–12 g).

Enzyme measurements. The demethylation of 3-methyl-4-monomethylaminoazobenzene[8] and the hydroxylation of zoxazolamine[9] by fortified rat liver homogenates were measured as previously described.

TABLE 1. URINARY EXCRETION OF L-ASCORBIC ACID OF RATS GIVEN MC BY DIFFERENT ROUTES OF ADMINISTRATION*

Route	No. of animals	Day	L-Ascorbic acid (mg/24 hr)
i.p.	16	5	9·2† (3·6–20·5)‡
	32	7	10·8 (4·9–28·0)
	8	12	18·5 (10·9–33·3)
	8	16	13·3 (7·7–22·6)
	8	22	10·6 (5·5–15·7)
	4	27	12·3 (6·5–16·6)
	4	35	10·5 (3·4–15·0)
	7	42	9·4 (4·5–12·8)
	3	49	10·8 (8·9–12·9)
	3	50	8·9 (6·2–10·9)
s.c.	2	1	0·7 (0·4– 1·0)
	6	2	1·8 (0·3– 3·8)
	6	5	7·1 (2·7–10·5)
	15	7	5·3 (1·9–12·1)
	7	13	6·4 (4·7– 9·2)
	4	17	8·4 (6·1–12·9)
	7	23	7·1 (5·5– 8·8)
	4	31	9·3 (5·7–15·7)
	4	38	9·3 (4·5–18·0)
	4	43	9·1 (5·1–17·7)
	4	45	9·0 (4·4–17·0)
	7	50	10·5 (4·3–16·6)
	3	60	11·1 (6·6–15·6)
	4	71	5·7 (4·0– 8·0)
	4	84	3·7 (1·7– 5·9)
	3	91	3·0 (0·7– 4·4)
	3	100	3·3 (2·0– 4·7)

* Rats given MC (10 mg/day) on days 1, 2, 3. Untreated or corn oil-administered rats excreted less than 1 mg of L-ascorbic acid in 24 hr.

† Average.

‡ Range.

RESULTS

L-*Ascorbic Acid Excretion*

Intraperitoneal or subcutaneous administration of MC produced a marked and prolonged increase in L-ascorbic acid excretion (Table 1). In contrast, oral doses of MC had only a small and transient effect. In the latter case peak levels were attained on day 5 (range 2·6–4·5; average 3·9 mg/24 hr); by day 7 these returned to near normal levels (average for 5 rats 1·9 mg). After subcutaneous or intraperitoneal injections of MC, L-ascorbic acid excretion exhibited a lag period, similar to that previously observed after intraperitoneal injection.[2] Maximal L-ascorbic acid excretion was achieved within a week after administration of MC and remained elevated for over seven weeks. During the first two weeks, intraperitoneally injected animals showed a significantly higher L-ascorbic acid excretion than the subcutaneous group, but subsequently the two groups excreted about the same amount of the vitamin.

Comparable increases of L-ascorbic acid excretion were observed in both male and female rats injected intraperitoneally with MC.

Studies of the effect of MC administration on free D-glucuronic acid excretion in rats were carried out by a chemical method. It was found that while L-ascorbic acid excretion was markedly enhanced, free D-glucuronic acid excretion increased only very slightly (Table 2). These results are in agreement with earlier isotopic studies[2, 5] which

TABLE 2. EFFECT OF MC, BARBITAL, AND CHLORETONE ON URINARY L-ASCORBIC ACID AND FREE D-GLUCURONIC ACID IN THE RAT

Rat no.	Compounds administered	Day	Ascorbic acid (mg/24 hr)	Day	Free glucuronic acid (mg/24 hr)
1	Corn oil i.p. on days 1–3	15	6·1	14	6·0
2	inclusive; 50 mg barbital	15	11·2	14	10·0
3	in 60 ml milk, days 9–15	15	4·6	14	5·2
4	inclusive	15	13·4	14	14·0
5	10 mg MC i.p. on days 1–3	15	11·6	14	3·3
6	inclusive	15	4·6	14	1·3
7		15	9·4	14	1·1
8		15	11·3	14	3·3
9		9	7·7	8	3·3
10		9	10·0	8	3·4
11		8	10·4	9	3·3
12	10 mg MC s.c. on days 1–3	8	6·7	9	2·7
13	inclusive	9	5·6	9	3·0
14	10 mg MC i.p. on days 1–3	15	18·4	14	26·9
15	inclusive; 50 mg barbital	15	28·4	14	22·2
16	in 60 ml milk on days	15	9·6	14	22·5
17	9–15 inclusive	15	12·0	14	12·0
18		15	10·0	14	15·5
19		15		14	25·3
20	10 mg MC i.p. on days 1–3	15	30·6	14	27·2
21	inclusive; 50 mg barbital	15	26·6	14	21·2
22	in 60 ml milk on days 1–15 inclusive	15	25·4	14	26·5
23	50 mg Chloretone in milk	3	36	4	2·9
24	on days 1–4 inclusive	3	10	4	3·4
25		3	9·5	4	3·4
26		3	30	4	3·2

Control free glucuronic acid excretion was 1·4 ± 0·5 mg (standard deviation).

indicated that MC stimulated the metabolic conversion of D-galactose-1-^{14}C and D-glucose-1^{14}C to labeled urinary L-gulonic and L-ascorbic acids but had only a small effect on their conversion to labeled D-glucuronic acid. In contrast to MC, barbital administration stimulated markedly the urinary excretion of both L-ascorbic acid and free D-glucuronic acid, although to a lesser extent than reported in previous studies.[5, 10, 11] The degree of this stimulatory effect was not diminished when MC and barbital were administered together. It was also determined that while Chloretone administration increased the urinary excretion of L-ascorbic acid, the output of free D-glucuronic acid increased only slightly; these results are similar to those obtained with MC.

Prolonged effect of MC on zoxazolamine hydroxylase activity

The subcutaneous administration of MC increased hepatic zoxazolamine hydroxylase activity within two days. The enzyme activity remained elevated for at least eight weeks after administration (Table 3).

TABLE 3. THE EFFECT OF SUBCUTANEOUSLY ADMINISTERED 3-METHYLCHOLANTHRENE ON THE ACTIVITIES OF LIVER 3-METHYL-4-MONOMETHYLAMINOAZOBENZENE DEMETHYLASE AND ZOXAZOLAMINE HYDROXYLASE IN RATS*

Day MC administered	Day of sacrifice	Enzyme determined	Corn oil-treated animals (average)	MC-treated animals (average)
1	2	Demethylase	16 (13 –19)	21 (15 –24)
1, 2	3	μg 3-methyl-AB	18† (18 –20)	23† (15 –30)
1, 2, 3	8	formed/12 min	15† (9 –22)	30† (27 –36)
1, 2, 3	24	per 50 mg liver	21 (20 –23)	30 (28 –35)
1, 2, 3	60		13 (8 –15)	20 (18 –22)
1	2	Hydroxylase	17 (14 –19)	23 (14 –28)
1, 2	3	μg zoxazolamine	17 (10 –20)	52 (44 –59)
1	3	metabolized/30 min	10 (7·5–14)	16 (4·4–30)
1, 2, 3	8	per 100 mg liver	17 (12 –22)	57 (38 –71)
1, 2, 3	24		16 (13 –20)	60 (53 –72)
1, 2, 3	60		18 (14 –24)	41 (31 –51)

* Male Wistar rats weighing 175–300 g were injected subcutaneously with 10 mg/day of 3-methylcholanthrene in 0·5 ml corn oil; 0·25 ml was injected on either side of the interscapular region.

† Eight animals in these groups; four in all others.

The enzyme system that N-demethylates 3-methyl-4-monomethyl-aminoazobenzene was stimulated to a much lesser extent. The animals used in the present study were older than those employed by Conney *et al.*[8] which led to the relatively high level of N-demethylase present in the livers of control rats.

It was found that large amounts of polar metabolites were formed by liver homogenates of rats treated with MC, whereas very small amounts were formed by control homogenates. These polar metabolites were not studied further, but they are probably hydroxylated derivatives of the azo dye substrate. These observations were made during the chromatography of the extracts from the incubated liver homogenates of azo dye metabolites on alumina.

The stimulatory effect of subcutaneously administered MC on liver microsomal hydroxylase activity thus showed a correspondence with the effect of the hydrocarbon on L-ascorbic acid excretion. The present results agree with earlier studies which indicated that drugs capable of increasing L-ascorbic acid excretion also enhance the activity of drug-metabolizing enzymes in liver.[2, 3] Further studies of the possible

TABLE 4. CONTRASTING EFFECTS OF INTRAPERITONEALLY ADMINISTERED 3-METHYLCHOLANTHRENE AND CHLORETONE ON LIVER ZOXAZOLAMINE HYDROXYLASE ACTIVITY AND URINARY L-ASCORBIC ACID IN RATS

Dose administered on first day, twice, 3–4 hr apart*	Time of sacrifice (days after first dose)	Liver zoxazolamine hydroxylase activity (μg substrate destroyed/ 30 m/100 mg) (average†)	Urinary ascorbic acid‡ (average†)
Corn Oil	1	3·1 (0·6– 4·8)§	0·55 (0·30– 0·85)§
	2	5·5 (0·6– 8·0)	0·81 (0·18– 1·6)
	3	4·7 (2·8– 6·9)	0·55 (0·34– 0·73)
	4	5·1 (0·3–10·6)	0·55 (0·36– 0·77)
MC 5 mg	1	29·1 (20·6–42·9)	0·53 (0·40– 0·68)
	2	38·1 (30·5–43·6)	0·95 (0·20– 1·8)
	3	40·5 (19·3–51·2)	2·8 (1·1 – 5·0)
	4	35·5 (25·8–43·3)	6·3 (2·3 – 9·6)
Chloretone 20 mg	1	10·0 (5·6–14·6)	5·9 (1·6 – 8·5)
	2	9·9 (4·7–13·8)	10·0 (2·9 –17·9)
	3	13·5 (6·9–17·0)	11·1 (7·4 –12·9)
	4	10·5 (7·4–14·5)	0·48 (0·27– 0·68)

* Each dose was given in 0·25 ml corn oil.
† Four animals in each group.
‡ Twenty-four hours preceding sacrifice.
§ Range.

relationship between these two effects revealed that they could be partly dissociated from each other. Thus, 24 hr after Chloretone administration, there was a marked increase in the urinary excretion of L-ascorbic acid but only a relatively small increase in zoxazolamine hydroxylase activity (Table 4, Fig. 1). On the other hand, 24 hr after the intraperitoneal administration of MC, zoxazolamine hydroxylase was markedly elevated, but there was no increase in urinary L-ascorbic acid excretion.

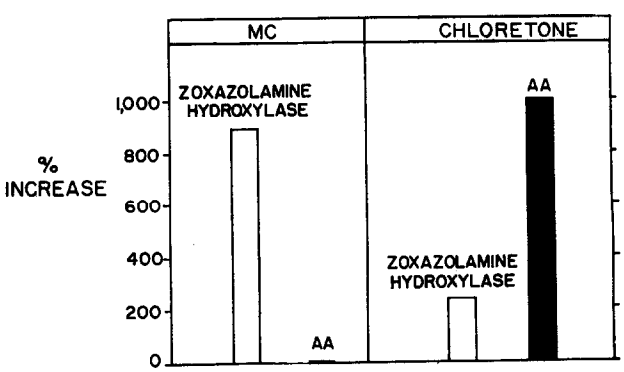

FIG. 1. Contrasting initial effects of MC and Chloretone on L-ascorbic acid excretion and liver zoxazolamine hydroxylase activity in rats.

TABLE 5. TISSUE DISTRIBUTION OF MC*

Wet weight (μg/g)

Route of administration	Day of sacrifice	Intraperitoneal fat†	Interscapular fat	Kidney	Liver	Spleen
oral	8	3·8 (2·4 – 6·4)		(< 0·02–0·05)	(< 0·15–0·21)	
i.p.	8	267 (72 –511)	**1·0 (0·75–1·3)**	3·8 (1·9 –5·5)	443 (214 –804)	**983 (370–1,680)**
	23	16·6 (10·8 – 25·8)		1·3 (1·0 –1·4)	246 (226 –264)	
	43	(0·75– 53)		0·12 (0·08–0·18)	77 (11·4–158)	
	53			**6·2 (5·3 –7·05)‡**	**115 (100 –141)**	**258 (63– 526)**
s.c.	8	**0·56 (0·31– 0·81)**		(< 0·02–0·36)	(< 0·15)§	(< **0·15**)
	53			(< 0·02)	(< 0·15)	(< 0·15)

* Ten mg/day of MC administered on days 1,2, and 3. Values corrected for recovery. Range of values in parentheses after average. Four to eight animals were used to obtain each average, except when indicated by bold type, in which case at least two animals were used. When lower limits are indicated, this was imposed by detectability.

† Consists of pooled retroperitoneal, renal, and testicular fat.

‡ The renal capsule was not removed in these experiments.

§ The concentration of the hydrocarbon as determined after administration of MC-6-^{14}C (New England Nuclear Corp.) was 0·02 μg/g; "metabolites" were 0·94 μg/g (12·9 g liver). "Metabolites" is that fraction not extracted from the hydrolysate by benzene. A single 30-mg dose was injected, prepared by mixing 5·4 mg MC-6-^{14}C (specific activity 9·5 μc/mg) and 24·6 mg of carrier.

Distribution of MC

Studies of the physiological distribution of MC in rats after intraperitoneal and subcutaneous doses showed that the prolonged biochemical effects of the hydrocarbon could be explained by its slow disappearance from the site of injection.

Examination of the peritoneal cavity with an ultraviolet lamp several weeks after the intraperitoneal injection of 30 mg of MC revealed an intense fluorescence due to the hydrocarbon. As long as 53 days after the intraperitoneal administration of MC, its presence could readily be demonstrated in liver, fat, and spleen.

It seemed probable that the MC measured in tissues included some free MC from within the peritoneal cavity present only on the surface of the tissues studied. To establish this point, four rats were given MC (10 mg/day i.p.) on days 1, 2, and 3. On day 8 the livers were removed, blotted once on tissue paper, and sectioned. The outermost layer of liver, about 1–3 mm thick, had an average concentration of 193 μg/g (range 108–258 μg/g), whereas the inner core had an average concentration of 22·1 μg/g (range 8·3–43 μg/g)*. Because of this finding, the entire organs (liver, spleen, etc.) were analysed. The amount of MC in the kidney was of particular interest because this organ is located posteriorly, is practically devoid of peritoneal covering, and has its own capsule. Thus kidney, unlike liver and spleen, would not be in good contact with the MC injected. The adrenals, when analysed on day 8, like the kidney contained low concentrations of the hydrocarbon (0·4 to 1 μg/g).

After the subcutaneous or oral administration of MC to rats, there were low concentrations of hydrocarbon in fat, kidney, spleen, and liver (Table 5). The present findings with orally administered MC are in agreement with previous studies by Dao and co-workers,[6] and Flesher and Sydnor.[13] After subcutaneous administration, MC remained at the injection site for a prolonged period of time and was slowly released. Even 53 days after subcutaneous injections the amount of MC at the injection site was measurable, and the values for three rats were 1·6, 8·8, and 9·0 per cent of the 30 mg injected. Fat from the injection site had a concentration of hydrocarbon averaging 97 μg/g (range for 4 rats 56–137 μg/g). The slow disappearance of MC from the subcutaneous injection site is similar to the findings of Heidelberger and Jones[14] with 1,2,5,6-dibenzanthracene and of Ballard and Nelson[15] with a steroid.

Rats which were given the hydrocarbon intraperitoneally died within 60–80 days, whereas the subcutaneously injected animals lived about twice as long and developed sarcomas at the injection site.

DISCUSSION

Subsequent to the reports[2, 3, 5] that MC and other polynuclear hydrocarbons increase the urinary excretion of L-ascorbic acid in rats, other workers[16, 17] have confirmed these findings. The present study demonstrates that this effect is long lasting and, as shown with subcutaneously injected animals, occurs with low concentrations of the hydrocarbon in liver. In this regard it is of interest that in an earlier study, polycyclic hydrocarbons were found to increase tissue levels of L-ascorbic acid.[18] In yet another study orally administered MC[19] resulted in increased adrenal ascorbic acid, which parallels the urinary excretion pattern observed in the present study.

* Experiments similar to those of Farah *et al.*[12] were carried out with the whole liver and benzene as the solvent. The concentration of MC in "runout" decreased within 5 min and increased thereafter, also indicating non-uniform distribution of MC.

In addition to the effects of MC on drug-metabolizing enzymes reported in this investigation, other biochemical findings of interest have been described. Inscoe and Axelrod[20] found that it increases glucuronyl transferase activity; Hollmann and Touster[16] noted that, in contrast with other compounds, it had no effect on uridine diphosphate glucose dehydrogenase and uridine diphosphate glucuronic acid (UDPGA) pyrophosphatase. These results led to a postulate that UDPGA might be converted to glucuronic acid via glucuronyl transferase and a glucuronidase,[16] and that stimulation of these reactions would increase L-ascorbic acid biosynthesis. The present findings—i.e. MC fails to stimulate free D-glucuronic acid excretion in rats but increases L-ascorbic acid excretion—cannot be explained by this mechanism.

Marsh and Carr[21] recently have shown that D-glucuronolactone is metabolized and excreted in the urine as D-glucaric acid, which in turn is an inhibitor of β-glucuronidase. These reactions will have to be considered in the explanation of the contrasting effects of MC and barbital on free D-glucuronic acid excretion.

The administration of MC *in vivo* was shown to increase the incorporation *in vitro* of ^{14}C-leucine into protein, and to augment binding capacity of microsomal proteins.[22] Gelboin and Sokoloff[23] have shown that MC administered to rats stimulates the conversion *in vitro* of soluble RNA-bound amino acids into liver microsomal proteins*. The observation[22] that MC increases demethylase activity is in contrast with our findings, (see Results).

It has been found recently that there are structural requirements for the induction of drug-metabolizing enzymes by polynuclear hydrocarbons[8, 26] and that they are different from those for carcinogenicity. In both cases, steric factors have to be considered,[26, 27] as well as electron transfer[28] which readily occurs with certain of these compounds, including MC.

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ADDENDUM

Subsequent to the submission of the present paper, a note[29] appeared on the metabolism of orally administered MC-6^{14}C in rats; the results reported are in agreement with previous[6, 13] and present studies.

* Touster and co-workers[24] interpreted their observation that ethionine blocks the effect of MC on L-ascorbic acid excretion by interfering with protein synthesis. It is therefore of interest that scurvy involves a decrease in protein synthesis. Martin *et al.*[25] observed that MC reduced the incidence of hemorrhage associated with scurvy but did not prevent the associated weight loss. MC also reversed some of the pathoses of scurvy.

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