CONNECTIVE TISSUE STUDIES[1]

II. The Effect of Vitamin C Deficiency on Healed Wounds

ARTHUR F. ABT,[2] SUSANNE von SCHUCHING and JOSEPH H. ROE

Radioisotope Service, Veterans Administration Center, Martinsburg, West Virginia

Received for publication May 11, 1959

In the chapter on the "History of Scurvy" in his monograph, *Scurvy, Past and Present*, Alfred Hess (1) presents an excellent summary. He notes that the ravages of scurvy have been recounted as historical occurrences since the campaigns of the Caesars, the pilgrimages of the Crusaders on land, and the voyages of the early explorers at sea.

In some of the early accounts, particularly that attributed to Richard Walter (2), who was chaplain to Lord Anson's expedition, the phenomenon is recorded which we have investigated and here present. Walter noted that the scars of wounds which have for many years healed were opened again when scurvy occurred. As an example, he describes an individual who had been initially wounded 50 years previously and whose wounds broke down and appeared as if they had never been healed when he contracted scurvy. A similar breakdown was described for a previously healed fracture. Lind (3) and Mead (4) are early writers who noted this breakdown of healed wounds following the occurrence of scurvy. Bourne (5) has reported the historical literature on scurvy and bone repair in his chapter on "Vitamin C and Bone". Recent reports on the effects of scurvy on healed wounds are sparse. Alan Hunt (6) in 1941 reported the effects of established scurvy on the healed wounds of three animals who had been deprived of all ascorbic acid from the twentieth day after operation. These studies were all histologic and showed changed staining qualities of the collagen of the scar. According to Hunt, "The ready reversion of the collagen of scars to its immature and weaker form offers an explanation of the breaking down of healed wounds in scurvy."

More recently, Pirani and Levenson (7) have made a histologic study of the effect of vitamin C deficiency in healed wounds. They performed midline laparotomy wounds and allowed them to heal for six weeks, during which period the animals were fed a nutritionally complete diet. They noted that after six weeks the wound scar was reduced to a thin line and was often hardly visible. In three instances where scurvy was produced, herniation developed

[1] This investigation was supported in part by Research Grant USPHS A-1949(C1) from the NIH, Public Health Service.

Presented in part at the Federation of Am. Soc. for Exper. Biol. meeting of the American Institute of Nutrition, April 15, 1959.

[2] Resident House Officer Johns Hopkins Hospital 1923–24.

at the site of the wound; however, complete dehiscence did not occur. Histologic observations on the scar tissue of the scorbutic guinea pigs whose laparotomy wounds had healed normally six weeks prior to the initiation of the scorbutic diet revealed fibroblastic proliferation and regression of connective tissue elements and hemorrhages. In an editorial comment (8) on the experiments of Pirani and Levenson it was noted that adequate ascorbic acid intake is essential not only for normal healing in the period immediately following trauma but also for the maintenance of previously formed scar tissue. Scar tissue seems to be more susceptible to vitamin C deficiency than is normal tissue.

We have studied the effect of scurvy on two groups of animals; the first were animals with recently produced wounds, and we wish to compare the data obtained from them (9) with data obtained from a second group whose original wounds had been allowed to heal for long periods of time.

In the study here reported, Carbon^{14} data obtained from the use of L-ascorbic-1-C^{14} acid (10,11) are included in addition to the chemical determination of ascorbic acid. Measurements of the pressure of wound rupture following the withdrawal of ascorbic acid are correlated with the amounts of ascorbic acid determined chemically in the blood and tissues.

MATERIALS AND METHODS

Guinea pigs of the Hartley strain, 220 to 250 grams, were obtained from the same source of supply. For the operational procedure a midline abdominal incision was made as previously reported (9). The animals were maintained on a Reid-Briggs semi-synthetic diet (12) with the addition of 1 gm. of ascorbic acid to 1 kg. of feed mixture, which furnished each animal with a 30 mg. daily ascorbic acid intake.

The control animals were maintained on this diet postoperatively for 70 to 78 days. The animals in which scurvy was induced were on the 30 mg. daily ascorbic acid intake for 43 days after operation, following which time the vitamin was completely removed from their diet. They were maintained on the scurvy-producing diet from 28 to 35 days before sacrifice. The total length of the postoperative period of the control and scorbutic groups was the same. The technique to determine the strength of the wound by measurement of the pressure required for wound rupture was the same as described in a previous paper (9). Three animals in each group were given an intraperitoneal injection of 5 mg. of radioactive ascorbic acid three to seven hours before sacrifice. Samples of blood and adrenal glands were analyzed from each animal to ascertain the nutritional status. Specimens of skin and muscle were removed from the scar as well as from the abdominal wall 3 cm. distant from the scar for analysis at sacrifice as previously described (9). Chemical determination of ascorbic acid was made according to the method of Roe and Keuther (13). The ascorbic acid was prepared according to the method of Salomon, Burns, and King (11) and the cyanide was prepared according to that of von Schuching and Enns (10). The radioactivity was determined by measuring the rate of ion production with an electrometer (14). The analysis of radioactive carbon was made by preparing $BaCO_3$ by the wet combustion method (15). The $BaC^{14}O_3$ from each sample was brought to a total weight of 400 mg. with inactive $BaCO_3$ and sealed with 4 gm. of silver chloride-lead chloride *in vacuo* under anhydrous condition in a Pyrex

test tube, 19 by 200 mm. These test tubes were tapered as they were sealed. $C^{14}O_2$ was liberated by heating at 400° for four hours (16) in an aluminum block which held 20 sample tubes. After cooling, the samples were attached to the ionization chamber by way of the elongated neck of the sample container which was easily broken following evacuation of the system. For our ionization chamber a CO_2 sample pressure of 36 mm. indicated total liberation of CO_2 from 400 mg. of $BaCO_3$. The ionization chamber was brought to atmospheric pressure by slow introduction of air through a drying tower. The method of preparation insured uniformity of samples. The L-ascorbic-1-C^{14} acid after conversion to $BaCO_3$ was assayed against a Carbon^{14} standard in a methane preflush flow counter operated in the proportional counting region.[3] The activity was 2 μc. per mg. One mg. of the labeled ascorbic acid gave a reading of 5.4×10^3 divisions per minute for our ionization chamber after conversion to $BaC^{14}O_3$.[4]

RESULTS

In Table I we have compiled data which will be used as a basis for comparison with succeeding experiments. It should be noted that observations obtained from a single injection of L-ascorbic-1-C^{14} acid containing 5 to 10 mg. of inactive ascorbic acid as a carrier are difficult to interpret, particularly when administered to a scorbutic guinea pig, because the values are increased in the scar as well as in the distant tissues. The values for the ascorbic acid content of the scar tissue show a greater increase than the values for the distant tissue in all of the groups, regardless of dietary intake or mode of administration. The scar tissue contains considerably greater amounts of radioactive Carbon^{14} than does the distant tissue, which is a finding similar to that obtained by chemical determination.

In a study presented in Table II, a midline abdominal incision was performed on a group of animals and allowed to heal for 72 days. At sacrifice the wounds could not be ruptured. The dietary intake was 30 mg. daily.

A second group of animals whose wounds had similarly healed showed a weight gain equal to the control group up to the time they were placed on the ascorbic acid-free diet. Thereafter, until the time of sacrifice, these scorbutic animals lost considerable weight, while the control group continued to make a slight weight gain until the time of sacrifice.

The days between operation and sacrifice for the animals in which scurvy was produced averaged 76 days, while a similar period for the control animal group averaged 72 days. The average number of days for the animals on the scorbutic diet was 33 days. In previously reported experiments dealing with the younger growing animal, average survival was 21 to 24 days and our present findings are in agreement with Slack (17) who noted that older animals developed signs of scurvy more slowly and at more individual rates.

[3] RCL Nucleometer Preflush Flow Counter and Scaler.

[4] Carbon^{14} activity represents essentially all radioactive ascorbic acid 24 hr. after injection (Salomon, L. L., Dissertation, Columbia University, 1952: and our own data).

TABLE I

Data are presented for short-term (10-day postoperative) abdominal wound healing. Values for both chemical analyses and C^{14} radioactive ascorbic acid tracer studies on groups of animals on varying vitamin C intake are presented. The chemical and C^{14} radioactive determinations were made following single injections of radioactive ascorbic acid. The groups of animals were maintained on three different dietary intakes of ascorbic acid for comparison. The first three groups of determinations were made on scorbutic animals. The next three groups of animals received 6 mg. daily intake of ascorbic acid, which is a maintenance level; and the final three groups were on a 30 mg. daily intake of ascorbic acid, which is a saturation level. Chemical determinations of ascorbic acid are expressed in mg. ascorbic acid per 100 ml. or gm. and C^{14} values are expressed in per cent of dose of L-ascorbic-1-C^{14} acid injected per ml. or gm. tissue. Analyses were made on whole blood, adrenal glands, scar skin and muscle, and distant abdominal skin and muscle.

Mg. Ascorbic Acid Intake Per Day	No. of Animals	Days Between Operation and Sacrifice	Whole Blood	Adrenals	Scar Skin	Distant Abdominal Skin	Scar Muscle	Distant Abdominal Muscle
			mg. Ascorbic Acid per 100 ml. or gm.					
Chemical Determination								
0 mg.	5	10	.09 ± .01	6.5 ± .90	2.5 ± .30	2.4 ± .70	.8 ± .10	.9 ± .10
0 mg. intake + 10 mg. L-ascorbic-1-C^{14} acid injected intraperitoneally 24 hours before sacrifice	5	10	.16 ± .01	—	6.9 ± 1.00	2.6 ± .32	3.2 ± .25	1.2 ± .13
			C^{14} Values in Per Cent of Dose per ml. of gm. Tissue					
C^{14} Determination								
0 mg. intake + 10 mg. L-ascorbic-1-C^{14} acid injected intraperitoneally 24 hours before sacrifice	4	10	.08	—	.06	.11	.26	.23
			.09	—	.15	.09	.37	.08
			.04	—	—	—	.60	.15
			.06	—	.09	—	.15	.08
		Average	.07	—	.10	.10	.34	.14
			mg. Ascorbic Acid per 100 ml. or gm.					
Chemical Determination								
6 mg. mixed in food	5	10	.16 ± .04	32.7 ± 6.00	5.5 ± .90	4.1 ± .40	3.0 ± .50	1.7 ± .20
6 mg. intake + 10 mg. L-ascorbic-1-C^{14} acid injected intraperitoneally 24 hours before sacrifice	5	10	.36 ± .01	—	15.8 ± 2.42	8.0 ± .72	8.4 ± .92	3.7 ± .32

			C^{14} Values in Per Cent of Dose per ml. or gm. Tissue					
C^{14} Determination								
6 mg. intake + 10 mg. L-ascorbic-1-C^{14} acid injected intraperitoneally 24 hours before sacrifice	5	10	—	—	.14	.06	.11	.03
			.04	—	—	.05	.07	.02
			.03	—	—	.04	.10	.02
			.04	—	.14	.19	.14	.08
			—	—	.30	.26	.29	.07
		Average	.04	—	.19	.12	.14	.04
			mg. Ascorbic Acid per 100 ml. or gm.					
Chemical Determination								
30 mg. by dropper	5	10	.22 ± .02	45.0 ± 7.80	6.6 ± .32	4.0 ± .43	3.7 ± .29	1.7 ± .18
30 mg. intake + 10 mg. L-ascorbic-1-C^{14} acid injected intraperitoneally 24 hours before sacrifice	2	10	.47	197.00	11.10	3.20	10.50	4.20
			.45	108.00	—	—	11.70	8.70
		Average	.46	152.00	11.10	3.20	11.10	6.45
			C^{14} Values in Per Cent of Dose per ml. or gm. Tissue					
C^{14} Determination								
30 mg. intake + 10 mg. L-ascorbic-1-C^{14} acid injected intraperitoneally 24 hours before sacrifice	2	10	—	3.10	.26	.22	.19	.36
			.20	1.60	—	—	.32	.61
		Average	.20	2.35	.26	.22	.26	.48

TABLE II

Study of Abdominal Wounds in Guinea Pigs Which Had Been Allowed to Heal for a Long Period (Approximately 70 Days Postoperatively)

Comparison of Weight, Pressure of Wound Rupture and Ascorbic Acid Content in Blood and Tissues in a Scorbutic Group and in a Group on a 30 mg. Daily Ascorbic Acid Intake.

Animal No.	Guinea Pig Weights in gm.			Days Between Operation and Sacrifice	Days on 30 mg. Ascorbic Acid Intake Following Operation	Days on Scorbutic Diet Prior to Sacrifice	Pressure Wound Rupture mm. Hg		Milligrams per 100 ml. (Whole Blood)	At Sacrifice				
										Milligrams Ascorbic Acid per 100 gm. Tissue				
	At Operation	At Start of Scorbutic Diet	At Sacrifice				Skin	Muscle		Adrenals	Scar Skin	Distant Abdominal Skin	Scar Muscle	Distant Abdominal Muscle
1	275	419	339	71	43	28	300	300	.12	10.50	5.47	2.20	3.11	.93
2	333	462	329	71	43	28	370	370	.10	15.00	3.18	3.44	1.38	.53
3	306	430	346	77	43	34	370	370	.03	3.40	1.10	1.52	.52	.32
4	359	472	364	77	43	34	350	350	.04	4.10	2.53	1.18	.57	1.53
5	360	437	451	78	43	35	290	290	.05	3.90	1.37	.85	.63	.28
							—	—						
							336*	336*						
6	326	575	396	78	43	35	Could not rupture		.12	9.90	3.08	2.41	2.83	.87
7	361	534	464	78	43	35	Could not rupture		.11	8.00	2.32	2.28	1.82	.79
Average...	331	476	384	76	43	33	(See above)		.08	7.83	2.72	1.98	1.55	.75
1	362	438	409	78	78	0	490	Could not rupture	1.27	184.00	8.97	5.64	5.97	2.76
2	320	450	540	73	73	0	Could not rupture		.46	117.00	6.43	5.34	5.15	1.67
3	364	514	541	70	70	0	Could not rupture		.51	110.40	5.95	4.22	4.85	1.63
4	352	502	496	70	70	0	Could not rupture		.63	78.20	6.67	4.14	3.94	1.95
Average...	350	476	496	72	72	0	—	—	.72	122.00	7.00	4.84	4.98	2.00

* Average.

In the scorbutic group the pressure for wound rupture for both the skin and the muscle averaged 336 mm. of Hg for the five individual pigs in which rupture occurred. In two pigs in the scorbutic group neither rupture in the skin or muscle layer could be produced.

In the control group a rupture of the skin layer was produced in only one animal and this occurred at a high pressure of 490 mm. of Hg. In none of the control group of animals could a rupture of the abdominal muscle layer of the scar be affected.

In the scorbutic group, the ascorbic acid level in the whole blood ranged from .03 to .12 mg. per 100 ml., with an average of .08 mg. per 100 ml. In the control group, the values ranged from .46 to 1.27 mg. per 100 ml., with an average value of .72 mg. per 100 ml. The average ascorbic acid content of the adrenals in the scorbutic group was about $\frac{1}{15}$ of the average content of the control group. In the scorbutic group the ascorbic acid content of the scar skin was 33 per cent greater than the distant abdominal skin, while the ascorbic acid content of the scar muscle was 100 per cent greater than the distant abdominal muscle. These differences are accentuated in the control group on the 30 mg. daily intake of ascorbic acid. Here we find the ascorbic acid content of the scar skin approximately 50 per cent greater than the distant abdominal skin, and the scar muscle shows an increase of 150 per cent over the distant abdominal muscle.

This significant increase in the ascorbic acid content of the scar skin and

TABLE III

C^{14} Determinations Calculated as Per Cent of Administered Dose of L-Ascorbic-1-C^{14} Acid in Blood and Tissues in the Two Groups of Animals Whose Wounds had been Allowed to Heal for a Long Period (Approximately 70 Days Postoperatively)

Animal No.	5 mg. L-ascorbic-1-C^{14} Acid Injected Intramuscularly Before Sacrifice	Days Between Operation and Sacrifice	C^{14} Values in Per Cent of Dose per ml. or gm. Tissue					
			Whole Blood	Adrenals	Scar Skin	Distant Abdominal Skin	Scar Muscle	Distant Abdominal Muscle
Scorbutic								
1	5 hr.	76	.001	6.7	—	—	.64	.45
2	3 hr.	76	—	2.2	.12	.16	.60	.16
3	3 hr.	76	.07	4.2	.17	.15	—	.07
Average....			.03	4.4	.14	.16	.62	.23
Control								
1	3 hr.	72	.22	3.4	.33	.19	.22	.06
2	6½ hr.	72	—	3.5	—	.11	.50	.12
3	7 hr.	72	.04	3.0	.19	.16	.16	.08
Average....			.13	3.3	.26	.15	.29	.09

TABLE IV

Ascorbic Acid Values of a Sample of Human Skin Obtained from a 30-Year-Old Lumbar Scar

Site of Scar	Duration of Scar	Ascorbic Acid, mg. per 100 gm.	
		Scar Skin	3 cm. Distant Abdominal Skin
Right lumbar area.....	30 yr.	9.0	5.5

scar muscle is similar to our results obtained in a group of younger animals in which only a 10 day interval existed between operation and sacrifice as previously reported (9).

Results of studies with L-ascorbic-1-C^{14} acid injected intramuscularly into the groups of animals whose wounds had been allowed to heal for a long period are noted in Table III. Here again the values for ascorbic acid in the scar tissue are higher than in the distant tissue.

Human Skin Analysis. In a sample of human skin from a 30-year-old lumbar scar the ascorbic acid content of the scar tissue was 64 per cent greater than a sample of normal skin tissue 3 cm. distant. The patient was on a high vitamin C intake before the specimens were removed.

DISCUSSION

We have confirmed experimentally in the guinea pig that wounds healed for a long period will break down in scurvy. This finding substantiates the observation of the physicians who accompanied the early explorations, as well as findings based on histologic studies on animals (6, 7).

From the fact that the previously healed wound in scurvy ruptures, we must conclude that ascorbic acid is essential for the integrity of scar tissue. In contrast, Elster (18) admits that ascorbic acid is essential for the formation of collagen, but notes that it may not be essential for the maintenance of preformed connective tissue.

We have noted that in scurvy the chemical values of ascorbic acid are at practically the same level in the scar as in the distant tissue for recently healed wounds in which all tissues are depleted in scurvy. A single injection of L-ascorbic-1-C^{14} acid to the scorbutic guinea pig brings out immediately the increased deposit of ascorbic acid in scar tissue.

We had previously noted the increased accumulation of ascorbic acid in recent scar tissue at various levels of vitamin C dietary intake. We wished to ascertain whether this increased accumulation would decline to the values found in an unoperated animal after a period of time had elapsed. Our results indicate that the higher level of ascorbic acid in the scar tissue persists both in the guinea pig and in man. We have demonstrated by the injection of L-

ascorbic-1-C^{14} acid that it contributed to the increased ascorbic acid level in the scar tissue, regardless of the age of the postoperative scar.

CONCLUSIONS

1) Abdominal wounds in the guinea pig which had been allowed to heal for a long period could be ruptured in animals after scurvy had been produced. Similar wounds could not be ruptured in animals on adequate vitamin C intake.

2) Adequate ascorbic acid intake is essential, not only for immediate postoperative healing, but also for the maintenance of previously formed scar tissue.

3) Ascorbic acid accumulates in scar tissue immediately following wounding and persists for long periods of time both in the guinea pig and in man.

4) The higher levels of ascorbic acid noted in scar tissue are derived in part from the recently administered ascorbic acid as demonstrated by single injections of radioactive ascorbic acid.

REFERENCES

1. HESS, A. F.: Chapter 1, History of Scurvy, *Scurvy, Past and Present.* J. B. Lippincott Co., Philadelphia, Pa., 1920, 1–22.
2. WALTER, RICHARD: *A Voyage Around the World in the Years 1740–44.* John Knapton and Paul Knapton, London, England, 1748.
3. LIND, R. J.: *Treatise on Scurvy.* S. Crowder, London, England, 1753, 151.
4. MEAD, R.: *Medical Works.* S. Crowder, London, England, 1762.
5. BOURNE, G. H.: *The Biochemistry and Physiology of Bone.* Academic Press, New York, N. Y., 1956, 500–555.
6. HUNT, A. N.: Role of vitamin C in wound healing. Brit. J. Surg., 1941, **28:** 458.
7. PIRANI, C. L. AND LEVENSON, S. M.: Effect of vitamin C deficiency on healed wounds. Proc. Soc. Exper. Biol. & Med., 1953, **82:** 95.
8. Editorials and Comments: Effect of vitamin C deficiency on healed wounds. J.A.M.A., 1953, **152:** 334.
9. ABT, A. F., VON SCHUCHING, S. AND ROE, J. H.: Connective tissue studies. I. Relation of dietary and tissue levels of ascorbic acid to the healing of surgically-induced wounds in guinea pigs. Bull. Johns Hopkins Hosp., 1959, **104:** 163.
10. VON SCHUCHING, S. AND ENNS, T.: Low pressure reduction of carbon^{14}-labeled barium carbonate to cyanide. J. Am. Chem. Soc., 1956, **78:** 4255.
11. SALOMON, L. L., BURNS, J. J. AND KING, C. G.: Synthesis of L-ascorbic-1-C^{14} acid from D-sorbitol. J. Am. Chem. Soc., 1952, **74:** 5161.
12. REID, M. E. AND BRIGGS, G. M.: Development of a semi-synthetic diet for young guinea pigs. J. Nutrition, 1953, **51:** 341.
13. ROE, J. H. AND KUETHER, C. A.: The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem., 1943, **147:** 399.
14. ENNS, T., et al.: Renal handling of glucose in dogs. Am. J. Physiol., 1959, **196:** 537.
15. LINDENBAUM, A., SCHUBERT, J. AND ARMSTRONG, W. D.: Rapid wet combustion method

for carbon determination, with particular reference to isotopic carbon. Anal. Chem., 1948, **20:** 1120.

16. ZWIEBEL, N., TURKEVICH, J. AND MILLER, W. W.: Preparation of radioactive CO_2 from $BaCO_3$. J. Am. Chem. Soc., 1949, **71:** 376.
17. SLACK, H. G. B.: Connective tissue growth stimulated by carrageenin. 3. The nature and amount of polysaccharide produced in normal and scorbutic guinea pigs and the metabolism of a chondroitin sulphuric acid fraction. Biochem. J., 1958, **69:** 126.
18. ELSTER, S. K.: Effect of ascorbic acid deficiency on collagen content of guinea pig tissue. J. Biol. Chem., 1950, **186:** 105.