of its glacial acetic acid solution with nitrous acid, it gives 2 isomeric mononitro-monobromo-*o*-cresols, melting at 90–91° and 120°, respectively. The latter has been proved to have the *para* structure.

2. The amino-cresols corresponding to these nitro compounds will be used in the further study of the migration of acyl from nitrogen to oxygen.

IOWA CITY, IOWA.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY, No. 378.]

THE QUANTITATIVE DETERMINATION OF THE ANTISCORBUTIC VITAMIN (VITAMIN C).

BY H. C. SHERMAN, V. K. LAMER AND H. L. CAMPBELL.

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Each vitamin is known by the physiological effects which result from its absence or from the intake of an insufficient quantity. These effects are failure of growth, or development of a deficiency disease, or both. The relative amounts of any one of the vitamins in different foods or in the same food before and after treatment can in general best be measured by feeding a suitable experimental animal, of standard initial age and size, with a basal diet adequate in all other respects but devoid of the vitamin in question, and finding how much of the food under investigation must be fed in order to supply enough of the vitamin to meet the needs of the standard test animal. This is sometimes determined in terms of the proportion in which the food under test when serving as the sole source of the vitamin in question must enter into the food mixture in order to render the food supply adequate in this respect. Or the food to be tested may be fed separately from the basal ration and the minimum quantity of the food which will thus suffice to protect the standard animal from vitamin deficiency may be determined in terms of the actual weight of food thus required as source of vitamin. This latter method is the one chiefly used in studies of the antiscorbutic vitamin and is sometimes referred to as determination of the minimum protective dose. See discussion by Chick and Hume.[1]

Holst and Frölich as early as 1912[2] made comparisons of the antiscorbutic properties of foods by finding whether or not certain arbitrarily chosen quantities which they fed would suffice to prevent the appearance of scurvy; but the basal diet which they employed, consisting usually of oats and water only, was deficient in other respects as well as in antiscorbutic vitamin.

Cohen and Mendel[3] devised a new basal ration planned to provide all necessary nutrients except the antiscorbutic, and showed that experimental scurvy can be induced in the guinea pig at will, and formulated more fully the criteria for the recognition

[1] Chick and Hume, *J. Biol. Chem.*, **39**, 203 (1919).

[2] Holst and Frölich, *Z. Hyg. Infektionsk.*, **72**, 1 (1912).

[3] Cohen and Mendel, *J. Biol. Chem.*, **35**, 425 (1918).

of this condition. Hess and Unger[4] employed chiefly a basal ration of oats, hay and water.

Chick, Hume, and Skelton,[5] Delf,[6] and other workers at the Lister Institute have used a basal ration of oats, bran and liberal amounts of autoclaved milk. They have done much to establish the importance of quantitative methods in the study of vitamin problems and have recorded a very valuable series of determinations of the antiscorbutic values of different foods; but it is doubtful whether the heat treatment to which they subjected the milk entering into their basal rations can be depended upon to destroy all of the Vitamin C and still leave the diet adequate as regards Vitamin A.[7]

Building upon the experience of the previous investigators as well as upon our studies of both rats and guinea pigs, we have still further developed the basal ration to ensure its freedom from Vitamin C and its entire adequacy in all other respects.

The basal diet as finally adopted is as follows.

	%
Oats, sound whole grain, ground in the laboratory as needed	59
Skimmed milk powder, heated on open trays at 110° until all antiscorbutic vitamin is destroyed	30
Butter fat, freshly prepared	10
Sodium chloride	1
	100

This diet supports excellent growth up to the time of the onset of scurvy.

Sound mature oats show no antiscorbutic property when fed to guinea pigs and are eaten readily by them. By using heated skimmed milk and fresh butter fat instead of heated whole milk, the absence of antiscorbutic vitamin is at least equally well ensured and the palatability of the diet and its fat-soluble vitamin content are improved. The heat treatment necessary to ensure complete destruction of Vitamin C in the skimmed milk powder should be determined by each investigator for his own material and technique, keeping in mind the likelihood of variation in antiscorbutic vitamin content of milk with the season and the importance of eliminating this vitamin completely from the basal ration without subjecting the food to such excessive heating as would give it a burnt taste and prevent its being eaten readily by the experimental animals. In our experiments, 2 hours' heating at 110° in shallow trays freely exposed to the air of the oven was found to be sufficient as determined by controlled feeding experiments.

After such heating the milk powder was of a light buff color. The butter fat, prepared by melting butter of good quality at the lowest possible temperature and freeing from water and curd by decantation and filtration through paper, was intimately mixed with the heated milk powder, the salt, and the freshly ground oats, so that the constituents

[4] Hess and Unger, *J. Biol. Chem.*, **35**, 479, 487 (1918).

[5] Chick, Hume and Skelton, *Lancet*, **1918**, II, 735.

[6] Delf, *Biochem. J.*, **12**, 416 (1918); **14**, 211 (1920).

[7] Delf, Ref. 6, pp. 436–42.

of the mixture could not be separated by the experimental animal while eating. The food mixture should be kept in a refrigerator in well-filled, air-tight containers; fresh portions fed daily, and feeding cups thoroughly cleansed at least twice a week, since the slightest incipient rancidity may result in failure of the animals to eat the food readily in which case the interpretation of results will be difficult and probably of very doubtful value.

Selection and care of experimental animals.—The experimental animals should either be bred by the investigator or purchased at an early and known age. They should be known to be growing at a normal rate. For about a week before the beginning of the experimental period they should be housed in the experimental cages or pens and fed with the above basal ration plus green food, both *ad libitum*. The experiment proper is then begun by simply discontinuing the green food with or without the feeding of a measured amount of antiscorbutic in addition to the basal ration. Our experience with about 200 of these experimental animals indicates that young guinea pigs 6 to 8 weeks old and weighing 300 to 350 g. are best used for this purpose. Placed at this age and size upon the above basal ration with food and water always available they usually eat about 18 to 20 g. of the dry food mixture per day and continue to grow for about 15 days, then lose weight rapidly and die of scurvy in from 26 to 34 days after being deprived of antiscorbutic food. If the animals are much younger, the results are somewhat less regular; if much older, they are somewhat less susceptible and also less desirable in that they are less likely to show good growth up to the time of onset of scurvy symptoms.

Symptoms, survival period, and autopsy findings.—With animals such as have been described, the first symptoms of scurvy appear after about 12 days on the above basal diet. The nature and sequence of the symptoms is so clearly described by Cohen and Mendel[3] and by Hess[8] as not to require discussion here. Loss of weight usually begins soon after the appearance of the first symptoms, though some animals begin to lose weight earlier. On the first or second day of the experimental period there may occur a decrease of body weight due to elimination of bulky intestinal contents from the green food of the fore-period. In such cases the minimum weight of the first or second day is taken as the initial weight of the experimental period. As an accurate weight-curve is helpful in the interpretation of results, each animal should be weighed at least once in 3 days throughout the experimental period. Table I shows the body weights at 3-day intervals of those of our experimental animals which were confined to the basal diet only.

[8] Hess, "Scurvy Past and Present," J. B. Lippincott and Co., 1920.

TABLE I

WEIGHT IN GRAMS, AT THREE-DAY INTERVALS, OF GUINEA PIGS ON BASAL DIET ONLY

Animal No.	Number of Days after Placing on Basal Diet											
	0	3	6	9	12	15	18	21	24	27	30	33
(80)	304	283	280	278	268	271	266	272	270	266	240	212
(12)	305	306	302	301	302	288	274	246	214	197	...	...
139	380	335	351	374	399	413	340	329	260	230	...	...
143	361	368	386	415	432	427	439	344	269	219	...	...
187	335	330	329	330	330	326	337	312	295	274	229	204
190	330	320	328	339	350	350	332	324	290	260	235	206
(191)	324	312	324	309	294	290	271	269	240	227	193	...
193	329	308	313	331	340	350	351	337	319	290	258	182
207	321	330	327	341	343	338	350	301	262	192	...	...
212	330	331	333	337	340	334	321	288	257	201	...	...
215	359	355	363	371	378	393	410	372	326	274	231	197
(216)	334	303	297	300	310	283	275	225	200	...	...	...
(217)	300	295	292	295	281	274	274	272	260	214	188	...
A29	300	313	325	341	352	357	358	350	312	255	215	...
A31	325	340	359	373	378	383	383	365	347	217	195	...
Av. of 15	329	322	327	336	340	338	332	314	275	236	220	200
Av. of 10	337	333	341	355	364	367	362	332	294	241	...	...

Fig. 1 shows a typical weight curve and one constructed from the average weights of the animals included in Table I omitting those whose numbers are enclosed in parentheses. Since the time of reaching a maximum

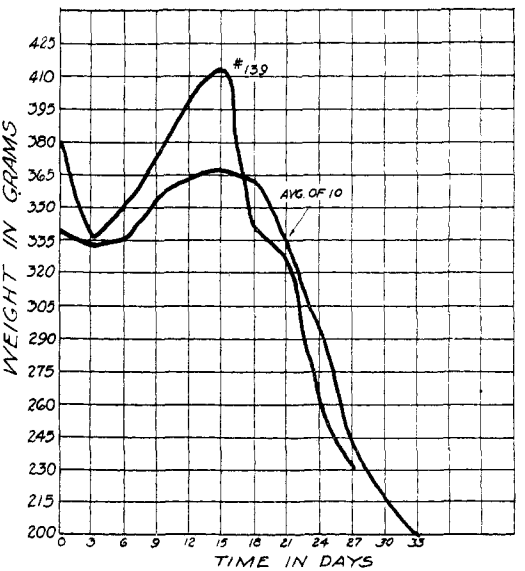

Fig. 1.

and beginning to lose weight differs somewhat with individuals, the effect of averaging the weights is to blunt the typical peak of the weight-curve. The animals whose numbers are enclosed in parentheses did not eat

so well as to show typical gains nor so poorly as to cause them to be rejected entirely.

The survival periods of the animals which we have kept upon the above basal diet only have been as follows: 33, 26, 27, 28, 34, 28, 27, 34, 26, 29, 32, 32, 32, 31, 34 days. At autopsy the findings which proved most

TABLE II

PROTOCOLS OF EXPERIMENTAL ANIMALS ON BASAL DIET ALONE OR WITH ANTISCORBUTIC

Animal No.	Tomato juice Cc.	Body weight Initial G.	Maximum G.	Final G.	Duration of experiment Days	Symptoms
143	0.0	361	439	219	28[a]	Very severe
193	0.0	329	351	182	34[a]	Very severe
207	0.0	321	350	192	28[a]	Very severe
173	1.0	312	405	269	90[b]	Very severe
184	1.0	321	384	255	53[a]	Very severe
90	1.4	332	394	201	41[a]	Severe
93	1.4	321	468	285	63[a]	Severe
92	1.5	332	368	285	91[b]	Severe
94	2.0	309	340	311	87[b]	Moderate
95	2.1	282	388	345	91[b]	Moderate
155	(*a*)	320	402	383	73[b]	Mild
181	(*b*)	323	503	475	85[b]	Mild
199	(*c*)	337	505	492	85[b]	Very mild
130	(*d*)	337	415	390	73[b]	Moderate

Autopsy Findings

Animal No.	Bony system Jaw	Teeth	Ribs	Joints	Hemorrhages Ribs	Intestine	Joints	Muscles
143	+++	+++	+++	++	++	++	++	++
193	+++	+++	+++	+++	+++	+++	+++	+++
207	+++	+++	+++	+	+++	++	++	++
173	+	−	+++	+++	+++	+	+	+
184	++	++	+++	+++	+++	+		++
90	+	+	tr	tr	+	+++	−	tr
93	+	+	+++	+++	+++	+	+++	
92	tr	tr	++	+		+		tr
94	−	−	+	++	−	+	++	tr
95	−	−	?	+	−	−	+	−
155	−	−	+	+		tr	−	−
181	−	−	?tr	−	−	tr	−	−
199	−	−	?+	−	−	+	−	−
130	−	+	++	+	−	+	+	+

(*a*) Received 3.9 cc. of tomato juice which had been heated for 1 hour at 100°; judged equal to 2.0 cc. of raw juice.

(*b*) Received 7.0 cc. of tomato juice which had been heated for 4 hours at 100°; judged equal to 2.5 cc. of raw juice.

(*c*) Received 4.0 cc. of tomato juice which had been heated for 4 hours at 60°; judged equal to 2.5 cc. of raw juice.

(*d*) Received 2.3 cc. of tomato juice which had been heated for 1 hour at 60°; judged equal to 1.75 cc. of raw juice.

[a] Animal died with scurvy.

[b] Animal was chloroformed for autopsy.

significant were: looseness of teeth, fragility of bones, enlargements and hemorrhages of joints and rib junctions.

Quantitative Expression of Results.—The results which we have obtained on feeding the basal diet alone and with the addition of filtered juice of canned tomatoes in measured amounts calculated to the basis of 300 g. of guinea pig per day may be summarized as follows.

With no antiscorbutic there is usually good initial growth followed by onset of scurvy symptoms, cessation of growth and great loss of weight before death from scurvy which occurs at 26 to 34 days. Autopsy reveals in severe form all the typical signs of scurvy, notably hemorrhages, fragility of bones, and looseness of teeth. (See Table II.)

With 1.0 cc. of tomato juice per day the duration of life is prolonged and becomes less uniform than on the completely scorbutic basal diet. The animals become lame and stiff before death and at death show severe hemorrhages, fragile bones and loose teeth.

With 1.5 cc. of tomato juice per day the animals usually live out the experimental period of 70 to 90 days after which it is unlikely that death from scurvy will occur. Scurvy symptoms develop with pain and stiffness in the joints and usually with loss in body weight. Hemorrhages and enlargements of rib junctions may become quite as pronounced as in the previous cases. (Since the animals live longer there is more time for these abnormalities to develop.) Fragility of the bones and looseness of teeth are less marked than when less antiscorbutic is given.

With 2.0 cc. of tomato juice per day growth after 15 days is subnormal and animals show soreness of joints without noticeable stiffness. When the animals are killed and examined after 70 to 90 days on this diet they show hemorrhages but not to a pronounced degree. Jaws and teeth appear normal and bones usually so.

With 3.0 cc. or more of tomato juice per day there is complete protection from scurvy as judged by examinations both during life and at autopsy. Growth is fully normal in all animals that eat the basal diet well. Hence 3 cc. appears to furnish a fully adequate allowance of the antiscorbutic vitamin.

Table II shows the protocols of typical cases of guinea pigs kept on the basal diet alone or with measured amounts of tomato juice as antiscorbutic. In all cases the volume of tomato juice as stated in the table is the amount which was fed per 300 g. guinea pig. This reduction of the dosage of antiscorbutic to a uniform basis of body weight of standard animal has been found to add considerably to the quantitative significance of the results. Following the precedent of Holst and Frölich the severity of autopsy findings such as hemorrhages and fragility of bones and looseness of teeth are indicated by, – (no different from normal),? (doubtful), tr (trace), and +, ++, +++ for increasing degrees of severity. From

this series of observations upon animals receiving no antiscorbutic vitamin and with different measured amounts up to the amount which affords complete protection and permits optimum growth it becomes possible to interpret the symptoms and autopsy findings in terms of the percentage of the required amount of antiscorbutic which was actually received by the animal in any individual case. This means that animals fed on this basal diet and receiving some antiscorbutic but not enough for complete protection can be given a quantitative rating based on the weight curve, survival period, and severity of the symptoms and autopsy findings. Thus in comparing the antiscorbutic properties of different foods or of the same food before and after treatment one is not confined entirely to a comparison of the so-called minimum protective dose since the quantitative rating of the degree of protection afforded permits comparisons to be made upon animals receiving less than the amount required for complete protection.

This method has been applied in studying the heat destruction of the antiscorbutic vitamin quantitatively, with feeding experiments to determine how much more of the heated juice must be fed in order to get the same result as with a known quantity of raw juice necessary to give the same degree of protection. This enables one to calculate the amount of antiscorbutic which had been destroyed by the heating.

The last four entries in Table II give the results of experiments of that kind. Following through the findings in the case of No. 155 and comparing them with results above and with the summary given previously, it was considered that on the whole 3.9 cc. of tomato juice heated for one hour at 100° showed practically the same antiscorbutic effect as 2 cc. of the unheated tomato juice and therefore that approximately $^1/_2$ of the antiscorbutic vitamin of the tomato juice had been destroyed by the heat treatment. The average of a considerable number of such experiments is practically 50%.

In the next case (No. 181) the animal received 7 cc. of tomato juice heated at 100° for 4 hours. The results indicated that this amount of heated juice was equivalent in antiscorbutic value to 2.5 cc. of raw juice, and the average of a number of such experiments indicated that heating for 4 hours at 100° resulted in the destruction of about 68% of the antiscorbutic vitamin present in the raw juice. The results of experiments on Nos. 199 and 130 (Table II, notes *c* and *d*) show results of heating at 60° with of course a lower rate of destruction than at 100°.

It is plain that in the same way one may compare the antiscorbutic potency of a measured amount of any other material with that of any of the different amounts of canned tomato juice representing any degree of protection up to the complete protection afforded by 3 cc. and can thus determine the relative amounts of antiscorbutic vitamin in different foods

both from experiments in which the exact minimum protective dose is found and from those in which there is a definite partial protection to which a quantitative rating can be given. This method possesses the advantage of the method of minimum protective doses, and in addition permits the use of a numerical value for each individual experiment of a series, the average of which should yield a more trustworthy result than when only those animals receiving exactly the minimum protective dose are taken into account. The data presented in the paper which follows will illustrate the application of this method.

Summary.

Relative amounts of antiscorbutic vitamin are measured by determining how much of the food under test is required to prevent scurvy in guinea pigs, or by a quantitative rating of the severity of the scurvy produced when less than this "minimum protective dose" is fed. This rating is based upon the weight-curve, duration of life, symptoms, and especially the autopsy findings of guinea pigs of standard initial age and weight. A new basal diet designed to furnish optimum quantities of all essential nutrients other than the antiscorbutic vitamin, and the technique of the experiments, are described.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY, No. 379.]

THE EFFECT OF TEMPERATURE AND THE CONCENTRATION OF HYDROGEN IONS UPON THE RATE OF DESTRUCTION OF ANTISCORBUTIC VITAMIN (VITAMIN C).[1,2]

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While the great instability of the antiscorbutic vitamin (Vitamin C) makes it an unpromising material for attempts at actual isolation, the development of methods for measuring relative amounts of this substance with considerable accuracy makes possible the study of its chemical behavior. The present investigation deals with the effects of heating for different lengths of time at 60°, 80°, and 100°, and at different known concentrations of hydrogen ion. As indicated by the earlier work of Delf,[3] the reaction by which Vitamin C is destroyed has a measurable

[1] The data for this paper are taken from a Dissertation submitted by Victor K. LaMer for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University, June, 1921. For the sake of brevity many of the details included in the dissertation are here omitted.

[2] Preliminary reports were presented at the January meeting of the Society of Experimental Biology and Medicine (*Proc. Soc. Exptl. Biol. Med.*, **18**, 122 (1921)) and the Rochester meeting of the American Chemical Society, April, 1921.

[3] Delf, *Biochem. J.*, **12**, 416 (1918).