III. EXPERIMENT

A. Subjects

Healthy young women were selected. Physical normality was judged on the basis of dietary histories, activity and health records, and the routine college entrance medical examination. Each prospective subject was interviewed and any personal inconveniences relative to the experiment were outlined to her at that time. The girl was made to feel the necessity for absolute cooperation if she volunteered.

B. Regimen

1. Basal diet

The basal diet was patterned after that used by members of the North Central States Cooperative Project ("Master" Project) and was planned to furnish approximately 70 mg. of ascorbic acid daily. The Thompson-Ohlson Table (1942) was used to estimate vitamin C content of foods and actual vitamin content was checked by chemical analysis. As a matter of convenience, meals were planned on a rotating five-day basis. The absolute meal plan was altered somewhat in the second year according to the availability of foodstuffs, but the basic dietary pattern did not change. Table 3 presents a

Table 3

Typical Menus As Used From 1943 to 1944

Breakfast (the same was served each day):

Orange or grapefruit juice	100 gm.
Whole grain cereal	100 "
Buttered toast	
Milk or cocoa	

Lunches	gm.	Dinners	gm.
Bacon	20	Meat	80
Tomato (raw)	40	Salad: Cabbage	50
Bread	60	Celery	30
Celery	30	Steamed potatoes	100
Peaches (canned)	75	Carrots	80
Hard-boiled egg (1/2)		Custard: Milk	120
Cookies		Egg (1/2)	
Milk		Milk	
Hamburger	50	Meat	70
Bun	40	Baked potato	100
Lettuce	30	Cauliflower	70
Dill pickle	20	Carrot (raw)	10
Peas	70	Celery curls	10
Egg (1)		Apple (raw)	100
Pastry		Milk	
Milk			
Fish salad: Fish	30	Tomato juice	100
Egg (1)		Meat	80
Celery, lettuce	90	Steamed potato	100
Pickled beets	20	Baked squash	100
Tomatoes (raw)	80	Cake	
Carrot (raw)	10	Milk	
Bread			
Apricots	75		
Milk			
Meat	50	Meat	70
Tomato conc.	30	Steamed potato	100
Peas	90	Beets	70
Lettuce	60	Raw rutabaga	20
Apple (raw)	100	Bread	
Bread		Custard: Milk	120
Hard-boiled egg (1/2)		Egg (1/2)	
Milk		Milk	
Corn casserole: Corn	70	Fish	60
Egg (1/2)		Baked potato	100
Milk	10	Stewed tomatoes	70
Lettuce	20	Cabbage (raw)	50
Pear (canned)	100	Doughnuts	
Milk		Milk	

typical set of menus used in the experiment from fall, 1943, to spring, 1944.

Each subject was required to drink a minimum of 500 gm. of milk daily, but larger amounts were allowed to individual subjects up to 800 gm. However, each subject was asked to drink a constant amount of milk each day. Frequent analyses of the milk available for use showed that it could not be depended upon as a source of vitamin C since it varied in vitamin content from zero to 2.1 mg. per 100 gm.

Whenever possible the same canning lot of a processed food was used during each experimental period. Many carbohydrate and fat foods were allowed ad libitum, including bread, butter, jelly, cereals, sugar, dried legumes, crackers, cookies, doughnuts, cake, pastry, rice, macaroni, cheese, gelatin, salad dressings, candy, tea, coffee, and soft drinks.

The experimental diet was carefully prepared in the college hospital kitchen under the direction of a dietitian. Food was weighed on a Chattilon scale reading in intervals of two grams. Meals were supervised by trained technicians.

To provide for any possible deficit in dietary nutrients, certain vitamin supplements were administered daily. The kind and dosage are listed in Table 4. This extra allowance of vitamins was planned to provide approximately the daily requirement of those vitamins as outlined by the National Research Council (1941). The dosage was sufficient to guarantee a generous intake and yet not lead to hypervitaminosis.

Table 4

Dosages of Vitamin Supplements
Administered Daily

Vitamin	Dosage	Frequency of administration
Betalin compound (Lilly)	One pulvule	Daily
Cod liver oil (White)	One capsule	Every other day
Riboflavin	One milligram	Daily

2. Activity

Subjects were asked to continue their ordinary routine of classes and extra-curricular activities. For example, if a subject had been accustomed to swimming several times a week, she was asked to continue that amount of swimming throughout the experiment. A careful record was made of any deviation from the usual pattern of exercise of the students, and unusual activity was discouraged.

3. Other restrictions

Subjects were requested to sleep and rest the same number of hours each day as far as college schedules permitted. Smoking and the use of drugs were not allowed. Mental and nervous stress were avoided, but it was impossible to control entirely the disturbing factors which are present in the lives of all young women today as, for example, movement of family and friends in the armed forces. In general, however, the group was well poised emotionally, and there was little

evidence at any time which would indicate otherwise.

C. Experimental Periods

Changes in experimental procedure were made from time to time as circumstances indicated. Therefore, some variation will be noted in experimental plan for certain subjects. Generally, the following plan was adhered to.

At the onset of the experiment subjects were placed on the control diet. When plasma and urinary values remained constant for ten days, an exercise period was begun after which the subject was allowed to recover on the control diet for a length of time sufficient to show any trend in plasma and urinary concentrations of vitamin C. Procedure after the recovery period depended on the amount of time available. Since each subject participated for one school quarter, experimental routine was limited by the length of the quarter and the amount of time required for the subject to reach a state of vitamin C equilibrium in the preliminary period. If time permitted, therefore, the recovery period was followed by a week to ten days during which the subject received 100 mg. of vitamin C daily to build up tissue stores. If, on the other hand, time was short, a saturation dose of 400 to 500 mg. of ascorbic acid was given orally to elevate tissue stores of the vitamin quickly. In either case, each subject received, after the recovery period, a supplementary dose of 100 mg. of

vitamin C daily for the duration of the experiment. An exercise and recovery period were repeated at this higher intake of vitamin. Throughout the study analyses were made three times weekly on the 24-hour urinary excretion and basal plasma concentrations of vitamin C. Dietary content of ascorbic acid was determined at intervals by chemically assaying the fruits, vegetables, and milk contained in it.

D. Exercise

The exercise was a stair-step type of procedure, one step 24 cm. high being used. The subject put either her left or her right foot on the step, lifted her entire weight to the top of the step with her other foot, and then returned to her original position. Each subject established the number of times she could comfortably reach the top of the step per minute, and music was played to help her maintain the rhythm of that pace.

Exercise lasted 15 or 22 minutes each day during the exercise period, two minutes of stair-stepping alternating with two minutes of rest. Exercise took place at some convenient time between four and six p.m.

Knowing the height of the step, the weight of the subject, and the number of times she reached the top of the step per minute, the energy expended was readily calculated.

E. Additional Observations of Subjects

Analyses were made three times weekly on hemoglobin, red blood cell count, and packed cell volume of blood. Basal metabolic rate was determined twice during the experimental period and additional records were kept of menstrual periods, incidence of illness, weight, and emotional tension.

F. Chemical Determinations

Chemical methods for the determination of ascorbic acid in biological fluids are based on the strong reducing power of the vitamin and particularly its reaction with the colored reagent, 2,6-dichlorophenol indophenol (Farmer and Abt, 1936; Bessey and King, 1933). Material to be analyzed by this technique is first extracted with an acid to eliminate interfering substances and to bring into solution and stabilize any vitamin C present (Bessey, 1938a). The standardized dye reagent is then titrated into the acid solution containing an unknown amount of ascorbic acid. The reaction which takes place is quantitative and rapid, and results, according to Bessey, in complete reduction of the indicator and the oxidation of ascorbic acid to dehydroascorbic acid.

The extracting agent which has more generally been used is metaphosphoric acid. It is not only an excellent precipitating agent but has a marked stabilizing effect on ascorbic

acid, delays the oxidation of the vitamin by catalysts as copper (Musulin and King, 1936) and has no effect on the dye (Bessey, 1938b).

Biological assay studies have confirmed the validity of this method (van Eekelen and Heinemann, 1938; Bessey, 1938a, 1938b). Other chemical methods as that used by Tauber and Kleiner (1935) are less specific or more time-consuming than the indophenol method (Bessey, 1938a).

Error may be introduced into this determination by possible interference from other dye-reducing substances, by colored or turbid solutions, and by failure to detect the faint titrimetric end point. These disadvantages have been eliminated somewhat by adapting the colorimetric analysis for use in the photoelectric colorimeter. The general principle of such an adaptation involves adding an excess of dye to a known amount of metaphosphoric acid filtrate and reading in terms of a logarithmic scale (Summerson, 1939). Summerson has said that the photoelectric method is more rapid, precise, and sensitive and has a higher degree of specificity than the visual technique.

Some investigators have recommended reduction of the acid extract of a material with hydrogen sulphide since a portion of vitamin C may be present in the reversibly oxidized form. However, King (1936) says that it is very likely that substances other than dehydroascorbic acid may be reduced and give a high end value for titration. Also the length of time

required for this procedure and the difficulty in removing the gas could easily offset any advantage in terms of information yielded, since loss of vitamin C could occur during the procedures concerned in removing the sulfide.

1. Plasma ascorbic acid

Plasma ascorbic acid was determined by the micro method of Farmer and Abt (1936). All blood samples were taken under basal conditions by finger puncture using lithium oxalate as an anticoagulant. Hemolyzed blood plasma values were not recorded owing to a possible effect of hemolysis on plasma vitamin C content. Plasma was deproteinized immediately and was titrated within one to four hours, being refrigerated meanwhile. Aliquots from two samples were titrated to obtain each end value.

Since plasma vitamin C determinations were made frequently it was not practical for the purposes of this experiment to use a macro method of determination, i.e., a method necessitating a venous puncture. One of the chief objections of workers in the field to the micro titration method has been the subjective quality of the end point, but Bessey (1938a) has stated that accuracy is generally within the range of biologic variation. Farmer and Abt (1936) report that the micro procedure has proved satisfactory for estimating vitamin C content of plasma and gives equally reliable values when compared with the macro method.

Table 3

Preservative Used per 24-Hour Sample of Urine.

Ingredient	Amount
	ml.
Sulfuric acid (5N)	100
Metaphosphoric acid (one per cent)	100
8-Hydroxy-quinoline (1.5 gm. per 100 ml. alcohol)	2

When each collection had been completed, it was measured and diluted to the nearest convenient volume with redistilled water. Samples were kept in a cool place when it was not possible to titrate them immediately. The titration values were read in the photoelectric colorimeter (Klett-Summerson) according to the method of Evelyn et al. (1938). An excess of dye was added to a one-milliliter aliquot of urine, and readings were taken at 10, 15, 20, 30, 40, 50, and 60 seconds from the time the dye was first added.

A correction for color and turbidity in urine was made by decolorizing the excess of dye in the sample after the timed readings had been completed. A final decolorized reading was obtained in the colorimeter and this value was subtracted from each of the original readings. Urinary readings were further corrected for the amount of dye necessary to bring an equivalent of the amount of extracting acid or preservative in a sample to the same end point. This value,

Comparative work in this laboratory has further established the specificity of the method. Simultaneous vitamin C determinations were made on two samples of blood, one analysis by the photoelectric macro technique of Mindlin and Butler (1938)[1], and one by the visual micro procedure of Farmer and Abt (1936). The values estimated for ascorbic acid content of blood plasma as determined from each method are given in table 5. Values for sample one were in agreement to three decimal places, and values for the second sample are well within the range of error for the chemical methods involved.

Table 5

Ascorbic Acid Content of Blood Plasma

Sample No.	Plasma Ascorbic Acid	
	Micro determination	Macro determination
	mg. per cent	mg. per cent
1	0.315	0.315
2	0.267	0.288

2. Urinary excretion of ascorbic acid

Urinary excretion of ascorbic acid was calculated from 24-hour excretion values. Collections were made in tightly stoppered brown bottles, and the kind and amount of preservative used are presented in table 6. Practically 100 per cent recoveries have been reported for ascorbic acid added to urine and stored 24 hours when this preservative was used[2].

1. Courtesy of Ardath A. Anders.
2. Courtesy of Dr. Williamina Armstrong Himwich.

termed a blank, was obtained by reading in the photoelectric colorimeter the decolorization of dye due to a one-milliliter aliquot containing a representative proportion of metaphosphoric acid and 8-hydroxy-quinoline as in the urine sample but no urine. The reducing value of urine alone was calculated by subtracting the blank reading from the original reading minus the decolorized reading.

The rate of drift of corrected readings for urine was then plotted against time and the free-hand curve extrapolated to zero time. A typical curve obtained in reading a sample of urine is shown in figure 2. Since reduction of dye by ascorbic acid is completed almost instantaneously and other reducing substances react more slowly, the reading at zero time should be an indication of vitamin activity alone (Evelyn et al., 1938). Evelyn et al. have pointed out that this extrapolation procedure is empirical, and while results cannot be more than approximate, errors are inconsequential when compared to a visual titration of urine where error may be several hundred per cent. These workers feel that if this technique is carried out accurately, relatively little error will result.

3. Food ascorbic acid

Ascorbic acid in food was determined by the modified Morell method of F. E. Deinze and M. S. Kanapaux (n.d.). One-fifth of the vitamin C-containing foods (fruits, vegetables,

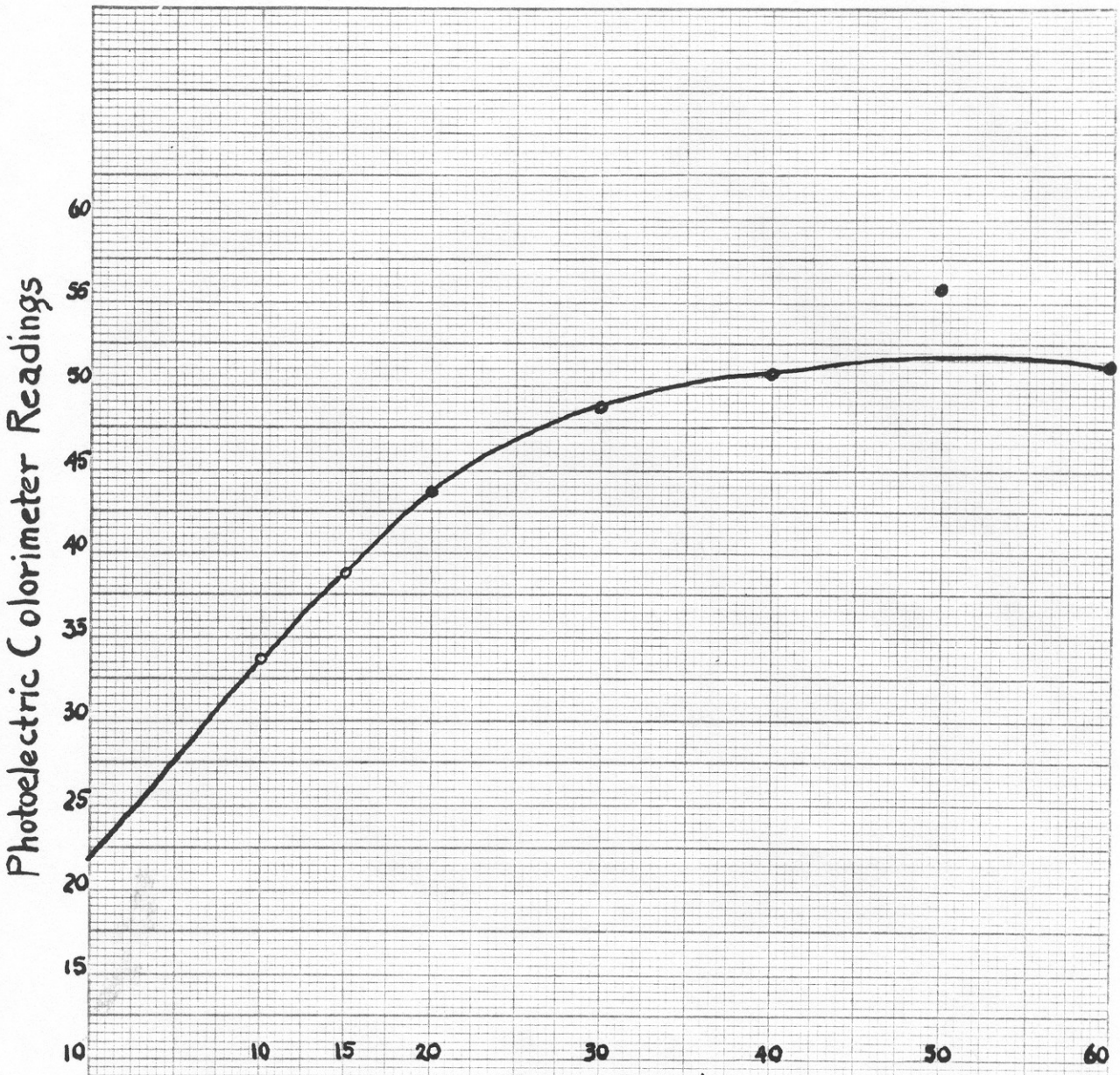

Figure 2. A Dye – Urine Reaction Velocity Curve Extrapolated to Zero Time

and milk) eaten in one day were collected each time the dietary was analyzed for vitamin content. Collections were made in brown bottles and the kind and proportion of preservative used are given in table 7. One per cent metaphosphoric acid was found to be entirely satisfactory since it gave a pH sufficiently low to prevent losses in the extraction process and yet high enough to prevent fading of the dye by acid (Loeffler and Ponting, n.d.).

Table 7

Preservative Used per 50 gm. of Food

Ingredient	Amount
	ml.
Metaphosphoric acid (one per cent)	350
8-Hydroxy-quinoline (1.5 gm. per 100 ml. alcohol)	1

Samples were added to the bottle just as the subject started to eat. Potatoes and milk were analyzed separately since the high turbidity found in these foods made handling difficult. Samples were refrigerated during the day. The Waring Blender was used to macerate the food and the resulting tissue extract was made up to a convenient volume and a few milliliters were filtered. One-milliliter aliquots were read in the photoelectric colorimeter 20 seconds after the addition of an excess of dye, since the end point of titration for vitamin C

is stable in 20 seconds for most plant tissue (Bessey, 1938a). The reading was corrected for color or turbidity and for reduction of dye by preservative by obtaining a completely decolorized and a blank reading as in the case of urinary analysis.

4. Standardization of dye

Dye was prepared every three weeks by dissolving a weighed amount of the dry product, sodium 2,6-dichlorobenzenoneindophenol (Eastman), in Sorenson's phosphate buffer solution and making up to a known volume. Dye was standardized once a week against pure ascorbic acid solutions of known concentration according to the recommendations of Todhunter[1]. The procedure used for standardizing dye varied according to the analysis for which the dye was to be used, a method adapted to the colorimeter being employed for use with food and urine and a method involving a visual titration for determining plasma ascorbic acid. Tables 8 and 9 list representative values obtained by both methods of standardization. Since dye solutions gradually changed in titer on standing, there was a considerable difference in standardization values from time to time, but readings for different ascorbic acid solutions on any one day represent a small percentage of error.

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1. Personal communication to Dr. Margaret A. Ohlson.

Table 8

Standardization[1] of Dye with Pure Ascorbic Acid Solutions of Known Concentration[2].

Date 1943	Scale Readings Corrected for Blank and Decolorized Reading: Solution No. 1	Solution No. 2	Solution No. 3	Deviation of Readings	Calculated Dye Factor	Remarks
				per cent		
6-14	106.00	107.50	106.00	1.4	.0004695	Fresh dye was
6-16	107.00	106.50	107.50	.9	.0004673	made up every
6-23	95.50	95.00		.5	.0005249	three weeks.
9-29	121.50	125.00	121.00	3.2	.0004082	Dye was
10- 4	124.00	128.50	131.50	5.7	.0003906	standardized
10-18	126.50	120.50		4.7	.0004049	once weekly.
11- 1	124.25	120.50		3.0	.0004086	
11-10	119.75	121.50	123.00	2.6	.0004118	
11-26	126.25	127.00	124.00	2.3	.0003976	
12-10	126.25	125.00	127.00	1.6	.0003966	
12-18	129.25	134.00	129.80	3.1	.0003816	
1- 6	116.50	118.50	122.50	4.9	.0004196	
1-13	121.75	124.00	121.50	2.0	.0004084	
1-20	105.25	106.40	108.90	3.4	.0004680	
1-20	116.00	112.50	115.00	3.0	.0004367	
1-27	111.75	112.25	113.00	1.1	.0004451	
2- 3	105.75	106.00		2.4	.0004722	
2-10	102.25	114.50	113.00	10.7	.0004549	
2-10	105.25	120.00	115.50	12.3	.0004402	
2-17	121.50	124.50	118.75	4.6	.0004113	
2-24	124.00	123.00	121.25	2.2	.0004073	
3- 2	121.00	128.00	127.25	5.5	.0003987	
3- 9	115.75	120.00	123.75	6.5	.0004173	
3-16	124.50	126.50	124.50	1.6	.0003995	

1. Standardization by use of photoelectric colorimeter for reading urine and food samples.
2. 5 mg. per 100 ml.

Table 9

Standardization[1] of Dye with Pure Ascorbic Acid Solutions of Known Concentration[2]

Date	Titration Value			Deviation of Readings	Calculated Dye Factor	Remarks
	Solution No.1	Solution No.2	Solution No.3			
	ml.	ml.	ml.	per cent		
6-16-43	1.14	1.13		.8	.011131	Fresh dye was
9-29-43	1.02	1.03		1.0	.01234	made up every
11- 1-43	1.26	1.41	1.33	10.0	.00947	three weeks.
12-10-43	1.46	1.46		0.0	.00866	Dye was
1- 6-44	1.07	1.05	1.01	5.6	.01196	standardized
1-20-44	1.12	1.12		0.0	.01128	once each week.
1-27-44	0.93	0.96		3.1	.01333	
2-10-44	0.97	1.04	1.03	6.7	.01256	
2-17-44	1.06	1.08	1.07	1.9	.01181	
3- 2-44	1.08	1.15	1.16	6.9	.01134	
3-16-44	1.17	1.17		0.0	.01079	

1. Standardization by titration for analysis of plasma ascorbic acid.
2. 5 mg. per 100 ml.

5. Blood tests

Hemoglobin concentration, erythrocyte count and cell volume were determined by the methods outlined by the Blood Methods Committee of the North Central States Cooperative Project ("Master" Project).

6. Special precautions

A complete list of solutions required for all analyses will be found in the appendix to this paper. Certain routine precautions are mentioned here.

Metaphosphoric acid was made up every two weeks in a ten-per cent solution which was diluted to the desired concentration at time of use. Metaphosphoric acid gradually changes to orthometaphosphoric acid on standing, but Bessey (1938a) states that it is satisfactory for use after 15 days.

All stock solutions were refrigerated when not in use. Water redistilled in glass was used throughout.

Corning filter No. 52 was used in making all readings in the photoelectric colorimeter. Green filters have a fine sensitivity over the lower range of concentrations of the vitamin C dye solutions to be read (Summerson, 1939).