A STUDY OF SOME OF THE FACTORS AFFECTING THE AMOUNT OF ASCORBIC ACID IN THE BLOOD PLASMA OF CATTLE

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Alfred Lee Bortree

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TABLE OF CONTENTS

INTRODUCTION

Under most conditions cattle are capable of synthesizing sufficient ascorbic acid for normal physiological functions. However, the work of Phillips (35)(36) indicated that reproductive failure, both in the male and female, may at times be due to insufficient ascorbic acid synthesis. The work of King and associates (82)(83)(85) with rats suggested the possibility of a relationship of the diet to ascorbic acid synthesis. A lipid fraction from alfalfa as well as many other compounds when fed to rats receiving a low ascorbic acid diet increased the urinary excretion of this vitamin. Ritz and coworkers (84) showed that the increased urinary output of ascorbic acid was due to a stimulation of synthesis of the vitamin.

This investigation was undertaken in order to study the level of ascorbic acid in the blood plasma of normal dairy cattle. The effect of various rations, organic compounds, and drugs on the blood plasma ascorbic acid level of cattle was also investigated.

REVIEW OF LITERATURE

The isolation and identification of ascorbic acid, commonly known as vitamin C, resulted from the systematic research of many workers. Waugh and King (1) were the first to identify the vitamin definitely. In 1932 these workers reported that the vitamin C as isolated in their laboratory was identical with the hexuronic acid prepared by Szent-Gyorgyi (2). In 1933 Szent-Gyorgyi and Haworth (3) named the substance ascorbic acid. Herbert and coworkers (4) established the structural formula of ascorbic acid using material obtained from adrenal glands by Szent-Gyorgyi. King (5) stated that Reichstein and workers succeeded in synthesizing the d- and later the l- form of the acid even before the structure was established with certainty. The first synthesis was carried out by treatment of l-xylose. Many different methods of synthesis have been devised, some of which are adapted to the commercial production of ascorbic acid.

The Determination of Ascorbic Acid

Methods

The most widely used methods for the quantitative determination of ascorbic acid are modifications of the method of Tillmans and associates (6). This method is based on the quantitative reduction of the indicator, 2, 6-dichlorophenol indophenol. Due to the many hazards and disadvantages involved in this method, numerous modifications and many entirely different methods have been devised but none of these have proven successful enough to supersede the methods based on the reduction of 2, 6-dichlorophenol indophenol. King (5) pointed out three hazards involved in this chemical determination; these were: (a) other substances may be present which reduce the titration reagent; (b) a portion of the vitamin may be present in the reversibly oxidized

form; and (c) substances may be present which interfere with the reaction of either the oxidizing or reducing agent. Several methods have been offered for the preliminary extraction of the material to be assayed; the only one that has been found to have definite advantages over the original trichloracetic acid extraction is the metaphosphoric acid extraction which has recently become the commonly used method. Kastlin and others (7) found that metaphosphoric acid protected vitamin C against atmospheric oxidation more effectively than other acids such as acetic or sulfuric.

Borsook and workers (8) reported a more complete recovery of ascorbic acid from tissue and plasma filtrates by precipitation with metaphosphoric acid than by precipitation with trichloracetic and sulfosalicylic acid.

Determination of plasma ascorbic acid

Mindlin and Butler (9) devised both a macromethod and a micromethod for measuring the reduction of 2, 6-dichlorophenol indophenol with the photoelectric colorimeter. This method has the advantages of eliminating, to a certain extent, the effects of reducing substances other than ascorbic acid in the plasma filtrate as well as errors inherent in visual measurements in colorimetry. The methylene blue method of Lund and Lieck (10) is based on the estimation of ascorbic acid by the decoloration of the dye in the presence of strong light. Roe (11) found that his method of determining ascorbic acid as furfural agreed, within experimental error, with the indophenol titration methods. This method consists of boiling the acid extract of tissue in which the ascorbic acid has been oxidized by passage through norite with HCl containing Sn Cl_2. The value obtained with HCl-$SnCl_2$ mixtures minus that given with HCl alone is the amount of furfural from ascorbic acid. Furfural is determined by the color formed with aniline stabilized with $SnCl_2$ and proper amounts of acetic acid. The dichlorophenol indophenol intradermal

test has been offered as a simple quick test for the ascorbic acid concentration in body tissues; however, Jetter (12) found no correlation between the fasting level of blood ascorbic acid and the decolorization time of the dye.

In checking the chemical method of determination by titration with the biological method, Lund and associates (13) found fairly good concordance. In the biological assay 0.7 milligram of ascorbic acid corresponds very nearly to the necessary addition of vitamin C to a diet deprived of all its vitamin C before hand.

Van Eekelen and Emmerie (14) criticized the generally accepted titrimetric procedures because of the error due to reducing substances other than ascorbic acid. In the method which they presented these substances were precipitated with mercuric acetate. However, their procedure has not been generally accepted due largely to the disadvantages involved in the complicated procedure being greater than any possible advantages.

Kastlin and investigators (7) found no advantage in the use of mercuric acetate as a precipitating agent for the removal of sulfur compounds from the filtrate.

Preserving ascorbic acid in blood samples

There are many conflicting opinions as to the best method of handling the drawn blood sample until it is ready for determination. Most of the data presented in the recent literature favors the procedure of holding the whole blood in the absence of light at a temperature approaching zero without adding a preservative. Greenberg and Rinehart (15), Kassan and Roe (16), and Borsook and coworkers (8) found that the red blood cells exerted a protective action against loss of vitamin C. In most cases, variations in the protective action were found with individual blood samples.

Barron and others (17) reported a close similarity in the rates of oxidation of ascorbic acid in blood serum and that in whole blood indicating that the presence of red cells had no influence on this oxidation.

Kassan and Roe (16) found that whole blood held at 3° C. remained unchanged in ascorbic acid content at the end of 52 hours. The advisability of using potassium cyanide, which has been reported to completely inhibit the catalytic oxidation of ascorbic acid, has been questioned by many workers and much evidence was presented which showed that there was no advantage in this procedure and in some cases it produced additional errors. Greenberg and Rinehart (15) and Wright and MacLenathen (18) reported that the addition of potassium cyanide to blood samples did not affect the results obtained.

Farmer and Abt (19) and Cushman and Butler (20) found that under certain conditions the use of cyanide invalidated the plasma ascorbic acid values due to decolorization of the dye by the cyanide. These workers found that the prevention of hemolysis of blood samples was much more effective in the reduction of losses of ascorbic acid than the addition of a preservative to the sample. The work of Greenberg and Rinehart (15) also illustrates this, as is shown in the following table.

Influence of Hemolysis and NaCN on Ascorbic Acid Values of Plasma

Blood Specimen	Time of Standing	Plasma without Hemolysis	Plasma - 0.1 cc hemolyzed blood per cc.	
			Without NaCN	With NaCN
	Min.	Mg. per cent	Mg. per cent	Mg. per cent
1	0	0.96	-	-
	5	-	0.73	0.73
	20	-	0.68	0.62
	40	0.90	0.62	0.57
	90	0.90	0.62	0.57
	120	0.85	-	-
2	0	1.10	0.75	0.75
	8	1.07	0.75	0.75
	20	-	0.70	0.70
	40	-	0.70	0.70
	60	0.93	0.64	0.64
	90	-	0.64	0.64
	120	0.87	0.58	0.58
	180	0.87	-	-

The results of Kassan and Roe (16) and Kastlin and others (7) showed that the stability of ascorbic acid was maintained in whole blood where the red cells were intact; but when the cell wall was broken the preservative effect upon the vitamin was lost and the oxyhemoglobin liberated caused oxidation of the ascorbic acid. Fujita and coworkers (21) found that the ascorbic acid was not oxidized by oxyhemoglobin but by oxygen liberated during the precipitation of the protein through the conversion of oxyhemoglobin to hematin. If the blood was treated with nitrogen, hydrogen, or carbon monoxide before precipitation no oxidation took place. This procedure was

used to estimate the ascorbic acid content of a number of tissues rich in hemoglobin. Lemberg and associates (22) studied the coupled oxidation of ascorbic acid and hemoglobin and found that oxyhemoglobin and ascorbic acid reacted directly to form choleglobin.

Physiological Functions of Ascorbic Acid

Formation of intercellular material

The functions of ascorbic acid have been studied by numerous investigators. Szent-Gyorgyi (23) stated that "ascorbic acid has, in all probability the simple function of giving off and taking up hydrogen atoms as do many other substances". Barron and associates (24) referred to ascorbic acid as a sluggish oxidation-reduction system protected in the body from oxidation by the ordinary oxidation catalysts and it seems to act as a promoter or catalyst of synthetic reactions - reduction and polymerization - thus taking a part in the building of cellular and intercellular structures.

The biological test for ascorbic acid is based upon the fact that this nutrient is necessary for the formation of all collagen or collagen-like substances essential to the development of intercellular material which prevents separation of the single layer of endothelial cells which form walls of blood capillaries and prevent characteristic scurvy hemorrhages. The importance of collagen in maintaining the body in the normal healthy state is further emphasized by the fact that it is the intercellular cementing substance which is the foundation of all fibrous structures, the matrices of bone, dentin, cartilage and all non-epithelial cementing substances.

Defense mechanism

Among the more important functions of ascorbic acid in the body is its function in the mechanism for detoxication. Most workers cite the beneficial effects of ascorbic acid in combating toxin producing substances without ex-

plaining the process. However, some workers believe that it is an oxidation or reduction process. Giroud (25) showed that there was a much higher mortality from spontaneous infection in rabbits on a low vitamin C diet as compared with those on a high vitamin C diet. Guinea pigs deficient in vitamin C do not form antitoxin against diphtheria toxoid as well as those receiving sufficient amounts of the vitamin in their diet. The possibility of this depressed formation of antibodies being an indirect effect due to inanition accompanying the deficiency was discussed.

Ascorbic acid has been used as a treatment for lead poisoning. Holmes and coworkers (26) found that in patients suffering from chronic lead poisoning the administration of 100 milligrams of ascorbic acid daily resulted in a marked improvement in vigor, cheerfulness, color of skin, and the blood picture. The patients also lost nervousness and irritability and no longer had tremors. The authors explained this effect as a reaction of the toxic lead ions with vitamin C to form a poorly ionized and much less toxic substance which is excreted in the feces. Leibowitz and Guggenheim (27) studied the detoxicating effect of ascorbic acid using potassium cyanide and phenol. They found that the potassium cyanide in minute amounts and in a slightly acid medium inhibited the oxidation of ascorbic acid in the air. However, this protective action became progressively weaker in higher concentrations and reached a point where additional cyanide accelerated the loss of the vitamin. It was found that there was a cyanide-ascorbic acid compound formed which was readily oxidizable and at the same time the cyanide lost its toxic effect on bacteria or mammals.

Phenol was also detoxified after short contact with the vitamin. However, saturation of the body with ascorbic acid does not prevent the toxic effect of the above compounds when they are taken in the free state.

Goldsmith and others (28) showed that there was a relationship between bronchial asthma and the ascorbic acid level in the blood of human beings. A determination of ascorbic acid in the blood plasma of 29 bronchial asthma patients revealed a wide variation in values, reaching a very low level in some cases. The values varied between 0.02 and 1.87 milligrams per 100 ml. of plasma. Two possible explanations for these low values were offered. During asthmatic seizures there may have been an increased requirement which the normal body supply could not meet, or the deficiency may be due to poor absorption from the gastrointestinal tract. Saturation of the body tissues with ascorbic acid appeared to have a beneficial effect by reducing the frequency and severity of the attacks.

Effect on appetite and metabolic rate

Several workers (29)(30)(31) have used Guinea pigs to study the physiological properties of ascorbic acid. It was found that the body reserves could be exhausted by feeding the animals a vitamin C free diet for ten to 12 days. When the body reserves of ascorbic acid became exhausted appetite was diminished and body weight decreased until death occurred. Anorexia was characteristic of ascorbic acid deficiency and as little as 0.1 milligram of ascorbic acid per day maintained a normal appetite in guinea pigs. The above workers compared the gaseous exchange of scorbutic guinea pigs with that of normal controls and concluded that a deficiency caused an increase in the metabolic rate. They offered as an explanation of these results the possible relationship between ascorbic acid intake and the activity of the thyroid. Daoud and workers (32) also studied the effect of ascorbic acid on metabolism. They found that with an increase in the metabolic rate there was an increased utilization of the vitamin as was evidenced by a decreased output in the urine. The maximum glycogenolysis caused by administration of

adrenaline was reached when the body of the subject was saturated with vitamin C by injection. These findings were not contradictory to those of Fidlar and others (30) because unless the utilization of blood sugar is interfered with by some other factor such as injecting adrenalin, saturation of the body with the vitamin has no noticeable effect on blood sugar.

Ralli and Sherry (33) in their experiments with normal and depancreatized dogs on a vitamin C free diet found that the urinary excretion and the fasting plasma level of ascorbic acid were markedly reduced in diabetic animals, indicating an increased utilization or a decreased synthesis of the vitamin.

Reproduction

That vitamin C may be a very essential factor in pregnancy was shown by the work of Ley (34). Ten women in whom two or more abortions had recently occurred were saturated with vitamin C in the early stages of their subsequent pregnancies and small oral doses continued until the sixth or seventh month of pregnancy. In every case a living child was delivered. The importance of ascorbic acid for reproduction in dairy cattle has been studied by Phillips and coworkers (35)(36) and will be referred to in connection with ascorbic acid metabolism in dairy animals.

Other functions

Sheppard and McHenry (31) observed that ascorbic acid was concerned directly or indirectly with water retention in the guinea pig. These workers also found that although scorbutic animals had a greater oxygen consumption the amount of fat retained was doubled.

Abbasy (37) found that vitamin C had a specific diuretic effect for human beings. He suggested that it may be of use where a mild diuretic, or

a slow and progressive dehydration of the body is to be desired.

Henry and Kon (38) studied the effect of vitamin C on the retention of calcium and phosphorus when calcium was fed to rats at a suboptimal level in the presence of adequate phosphorus. Their results showed that the addition of vitamin C did not improve the retention of either element.

Ascorbic Acid Requirement of Man

Experiments conducted to produce scurvy indicated that the tissues of the human body serve as a source of sufficient vitamin C to prevent scurvy for a relatively long period of time. Crandon Lund and Dill (39) found that ascorbic acid in the blood plasma of patients fed a vitamin C free diet dropped to a low level in ten days and disappeared from the plasma in 30 days. The plasma level was zero for 13 weeks before the first clinical evidence of scurvy appeared. Definite failure of wound healing did not occur until six months after the experiment started. It was observed that there was a closer correlation between the ascorbic acid content of the white cell-platelet layer and the development of disease than existed between the plasma level and the development of disease. These findings substantiate the suggestion of Lonzer (40).

Daum and associates (41) observed that there was very little agreement between the pathological state and the blood ascorbic acid except in the case of infection with elevated temperature where values were consistently low. They concluded that the blood level was no indication of the body's need, depletion, or saturation. Wortis (42) and others found that a normal blood level of ascorbic acid was invariably associated with normal spinal fluid content and a normal urinary excretion. When the blood level was subnormal the spinal fluid content and urinary output were also subnormal. Kastlin and coworkers (7) in studying ascorbic acid deficiency found that infection

and toxic conditions may increase the metabolic requirement. Low gastric acidity and delayed assimilation may permit oxidative loss of the vitamin after ingestion. Decreased intestinal absorption may also subject the vitamin to destructive bacterial action.

Drigalski (43) studied the vitamin C content of the blood of men on a diet poor in vitamin C. The values ranged between 0.2 and 0.7 milligrams per cent with spontaneous variations in the blood picture of the individuals on a constant regime.

Concentration of Ascorbic Acid in Various Organs

Suprarenals

Many investigators have studied the concentration of ascorbic acid in the various organs of the body in an attempt to determine the functions and possible site of ascorbic acid synthesis. Phillips and Stare (44) determined the quantity of reducing substance in the tissues of fluorine fed cows. They found that the supararenals, the suprarenal cortex and the anterior lobe of the hypophysis were exceptionally high in vitamin C in both the experimental animals and the controls.

The distribution of ascorbic acid in the adrenal gland has been studied by Glick and Biskind (45) and Westergaard (46), using the silver nitrate staining technique. The fascicular region was found to have the highest ascorbic acid concentration at all stages of development. Beznak and Hariss (47) and Harris and Ray (48) showed that the adrenals do not act as a storehouse for the vitamin and that adrenalectomized rats remained healthy on a vitamin C-free diet. They concluded that this evidence is opposed to the theory that the suprarenal controls synthesis of the vitamin. These workers explained the high concentration in the suprarenal cortex as being involved in a system needed to maintain adrenaline-like substances in a reduced condition.

Pituitary

Glick and Biskind (49) who made a study of the concentration of vitamin C in the pars nervosa, pars intermedia and the pars distalis of beef animals concluded that the pars intermedia was the most potent tissue source of ascorbic acid. Biological assay values checked their titration values very well. Giroud and workers (50) found the concentration of ascorbic acid in the pituitary was only slightly affected with the development of scurvy in guinea pigs and even when the disease had reached full development this body remained relatively high in ascorbic acid.

Leblond and Chamorro (51) reported that the vitamin C concentration in the liver and heart was decreased on hypophysectomy while that in the kidneys and muscles was increased. In studying the point of synthesis of ascorbic acid it is of interest to note that these workers found that hypophysectomized rats were capable of synthesizing ascorbic acid. Bowman (52) and associates, however, found a marked decrease in the ascorbic acid values of the liver, kidney adrenals and small intestines of hypophysectomized rats.

Thymus

Glick and Biskind (53) found that the thymus was unlike other organs in that the concentration of vitamin C decreased with increased maturity of the animal due to a displacement of glandular tissues by other tissues practically devoid of the vitamin.

Corpus luteum

The concentration of ascorbic acid in the corpus luteum remains high during pregnancy. According to Biskind and Glick (54) it contained 1.4 milligrams ascorbic acid per gram of tissue when the organ was most fully developed. These investigators were of the opinion that vitamin C is unrelated

to the female sex hormone estrin since follicular fluid is low in ascorbic acid and the estrin content of the corpora lutea of gestation decreased to almost zero towards the end of pregnancy, although the vitamin C remains at a high level. However, the variations of the vitamin C content of the corpus luteum seemed to parallel the progesterone content.

Intestine

Glick and Biskind (55) found a rather high constant concentration of ascorbic acid in the mucosa, and a much lower concentration in the submucosa and muscle of the intestine.

Embryo

Ray (56) was unable to find titratable amounts of ascorbic acid in the chick embryo until after the fourth day of incubation. The concentration increased as the incubation period progressed. Preliminary experiments performed by injecting ten times the amount of ascorbic acid present in the developing embryo, into the air space of eggs before incubation gave totally negative results. Barnett and Bourne (57) concluded that ascorbic acid is present in too small quantities in the early extra-embryonic tissues to be determined by ordinary methods of quantitative analysis. At the fourth day deposits of the vitamin appeared in a variety of tissues within the embryo, including parts of the central nervous system, part of the gut wall, some of the somatic material, and certain mesenchymatous cells. Pincus and Werthessen (58) found that ascorbic acid had an inhibitory effect on mammalian ovum growth in vivo, which they explained on the basis that the glutathione was used to maintain the ascorbic acid in the reduced state and was not available to stimulate the blastocyst expansion and glandular proliferation.

Mechanisms in the Body for Conserving Ascorbic Acid

Glutathione

The recognized close relationship existing between ascorbic acid and glutathione in the body has resulted in many studies of the interrelationship of these two substances. In 1936 Hopkins and Morgan (59) reported that when ascorbic acid and glutathione were together in the presence of certain oxidizing agents the glutathione completely protected the vitamin from oxidation. The glutathione which protects ascorbic acid from oxidation was oxidized at the same rate as ascorbic acid alone would have been oxidized in the presence of the same concentration of enzyme. Only when the glutathione had practically disappeared from the system did the oxidation of ascorbic acid begin. It was also stated that glutathione completely protected ascorbic acid from oxidation by copper catalysis and that the mechanism of this protection must be different from that which operates in the case of the enzyme. In the latter it depends upon hydrogen transference, in the former on inhibition of the catalyst. Crook and Hopkins (60), and Kertesz (61) were able to reproduce these results and in addition the latter found that ascorbic acid and glutathione in the absence of metallic or other catalysts were not oxidized by atmospheric oxygen. Kertesz (61) also studied the effect of pH and found that glutathione protected ascorbic acid from oxidation at pH of 7.4, and both ascorbic acid and glutathione were oxidized simultaneously. Borsook and Jeffreys (62) made a very thorough study of the protective action of glutathione on ascorbic acid at various concentrations and over a rather wide pH range. They found that at a pH of 5.5 and higher, and at a temperature of 37° C. the reversibly oxidized form of ascorbic acid undergoes an irreversible change whereby it loses its antiscorbutic potency and the property of convertibility to a reduced form by hydrogen sulfide. It

was also noted that this change is not an oxidation since it proceeded as quickly in vacuo as in contact with air. Since this irreversible change proceeded rapidly at the pH of body fluids and body tissues it was concluded that the vitamin was protected from this change in vivo. Their experiments on blood, urine and tissue slices showed that this protective action involved the reduction of the reversibly oxidized vitamin and that this could be affected rapidly by glutathione when it was present in sufficient concentrations. Their in vitro experiments showed that glutathione, when present in proportions equal to those found in such body tissues as the liver and adrenals almost completely reduced the reversibly oxidized ascorbic acid. Castex and Schteingart (63) studied the ratio of glutathione to ascorbic acid in blood and observed that disease altered the normal ratio. In general when ascorbic acid values were low the glutathione values were high. Yamada (64) used sulfur to increase glutathione synthesis in rabbits and albino rats. Sublimated sulfur given orally, colloidal sulfur given intraperitoneally, and a sulfur paste smeared on the inside of the ear greatly increased the glutathione content of the tissues except in the small intestine where there was a large decrease.

Svirbely (65) reported that cysteine, cystine and glutathione played a part in this protective mechanism. Mawson (66) substantiated the opinion of Svirbely but stated that liver extracts from which glutathione had been removed by prolonged dialysis still retained the protective properties of fresh extract. He was of the opinion that there is a chain of protective mechanisms in animal tissues which function by inhibiting metallic catalysts which are present in body fluids in such concentrations that without a protective mechanism of some kind, ascorbic acid could never exist in them.

Purines, creatinine and creatine

The influence of purines, creatinine and creatine on the oxidation of vitamin C was studied by Giri (67) and xanthine, uric acid, and theophylline were shown to completely inhibit oxidation by copper while caffeine and theobromine had no influence on the oxidation and it was concluded from this work that the free imino group exerted the influence of the purines, as those purines with the imino groups methylated showed no inhibiting effect. It was also shown that creatine had no influence on the oxidation while creatinine exerted a powerful protective action against the oxidation of the vitamin.

Mystkowski and Lasocka (68) classified the protective mechanisms of blood serum in two classes. The class which inhibits conversion of ascorbic acid to dehydroscorbic acid by binding copper, includes proteins, amino acids and salts. The second class consists of glutathione and possibly other thiol derivatives which prevent conversion of dehydroascorbic acid into further irreversibly oxidized products.

Sodium chloride

Mystkowski and Lasocka (68), Kellie and Zilva (69) and Høygaard and Rasmussen (70) reported that sodium chloride protects ascorbic acid. It appears that only the chlorides exert the retarding influence on oxidation.

Tissue extract

Kellie and Zilva (69) studied the protective action of guinea pig tissues by adding the distilled water extract of one gram of tissue to a solution containing 15 mg. of ascorbic acid and making the solution up to 40 ml. The following results were obtained.

Milligrams Ascorbic Acid per Hundred Milliliters of Water

Hours	Liver	Kidney	Muscle	Spleen	Plasma	Distilled Water
0	38.2	37.5	37.0	37.5	36.8	32.0
1	37.7	36.3	36.1	37.4	36.1	24.9
2	37.4	35.6	35.1	37.0	35.4	15.2
3	37.0	34.7	34.6	36.7	35.2	9.8
4	36.8	34.2	33.7	36.3	34.6	5.3
20	31.1	25.6	28.8	23.5	24.0	0.0

It was demonstrated that the protective effect shown by these data was due to inhibiting the catalytic action of metals.

Bacteria

Esselen (71) reported that fast growing types of bacteria were very effective in inhibiting the in vitro oxidation of ascorbic acid.

Ascorbic Acid Synthesis

Precursors

Experimental evidence has been presented (72) which pointed toward mannose as a possible precursor of ascorbic acid. Hawthorne and Harrison (73) found no evidence of this using minced rat liver incubated in ringers-phosphate buffer solution in the presence of oxygen. Results from the intravenous or subcutaneous injection of mannose into rats were also negative. In Rudra's (72)(74) studies of certain sugars as precursors of ascorbic acid and the influence of manganese on the synthesis, he found that a concentration of 0.001 to 0.0005 per cent manganese was most favorable for the synthesis of ascorbic acid in vitro. The hypothesis was advanced that the inability of the guinea pig and man to synthesize their requirement of ascorbic acid is due to a lack or insufficiency of manganese in their tissues.

Synthesis

Due to the marked variations in the ascorbic acid content of the small intestine under various conditions many workers are of the opinion that synthesis takes place in this tissue.

Hopkins and Slater (75) studied the effects of incomplete diets on the ascorbic acid in the small intestine and liver of rats. They are of the opinion that the liver and the intestine synthesize the vitamin and that there is a mechanism through which a mutual adjustment in the productive activities of these organs is established. They concluded that there are many precursors of ascorbic acid and that the cells of the epithelium of the intestine will use protein and fat in the absence of carbohydrate, as a source of a precursor for the synthesis of ascorbic acid. Zilva (76) did not agree with the results or the hypothesis of Hopkins and Slater. He questioned that the increase in reducing power of the liver and intestines under the conditions of the experiment was due to ascorbic acid. He was of the opinion that if the increase in reducing power was due to ascorbic acid such an increase could be assumed to be due to the transfer of the vitamin from other parts of the body to the organ in question in order to fulfill a physiological need conditioned by the constitution of the diet.

Excretion of Ascorbic Acid

Ralli and others (77) studied the mechanism of excretion of vitamin C by the human kidney and reported that the data indicated that vitamin C is excreted only by filtration, that it is actively reabsorbed by the renal tubules and that the factor which limits this reabsorptive process is the existence of a maximal rate. From this it was concluded that the quantity of vitamin C excreted in the urine is determined by the plasma concentration of the vitamin, the rate of glomerular filtration, and by the maximal rate of

tubular reabsorption. The work of Sherry and associates (78) supported the above report. In addition these investigators were of the opinion that the reabsorption of vitamin C and of glucose does not involve a common mechanism. There are many factors that affect the rate of ascorbic acid excretion. Ether anesthesia causes a marked increase in the urinary output of ascorbic acid (79)(80)(81). In cases of renal scurvy, doses of 500 milligrams of ascorbic acid do not have a permanent effect upon the plasma level of ascorbic acid due to the rapid rate of excretion.

Factors Affecting the Excretion of Ascorbic Acid

Stimulation of ascorbic acid synthesis

Recent work showed that a high urinary excretion of ascorbic acid may be produced by stimulating synthesis in those animals capable of ascorbic acid synthesis. Musulin and coworkers (82) found that oats, oat oil, the unsaponifiable material of oat oil, and of halibut liver oil quickly induced a high rate of excretion of ascorbic acid in the urine of rats. Certain volatile fractions from liver oil and oat oil were found to be especially active while fatty acids and common sterols were inactive. The urine ascorbic acid was measured by direct titration with 2,6-dichlorophenol indophenol and verified by guinea pig assays for vitamin C.

These workers suggested the possibility of the lipid effect being exerted through an indirect agency such as accelerating a synthesis from other substances or by serving as a protective agent against tissue destruction of its vitamin. Longenecker and associates (83) continued this study and found that the unsaponifiable fractions from alfalfa and lawn grass in addition to those from oat and fish oils were effective in raising the urinary output of ascorbic acid by rats. An increase from about 0.3 milligrams per day to 10 to 15 milligrams per day was observed. The above workers also

showed that the effect of feeding certain cyclic ketones was even more pronounced. Both d- and l-carvone, isophorone, pulegone and thujone were among the more effective ketones in increasing the excretion of the vitamin in urine. Ritz (84) and coworkers studied the effect of feeding carvone and salicylates on the urinary excretions of ascorbic acid by rats. They found that both these compounds increased urinary output, but two entirely different mechanisms were involved. The salicylates increased the loss of ascorbic acid from the tissues as was shown by analysis of the brain and liver of animals given doses of 30 milligrams per 100 grams of body weight twice a day for three days. The livers, plasma and to a slight extent the brain of animals receiving the same dosage of carvone showed a consistently higher ascorbic acid content than controls along with an increased excretion which was twice that of the salicylate treated rats. The authors concluded that there was an increased production in the liver which is directly related to detoxification of the terpenes at this point. Studies were also made with nephrectomized rats which indicated that the effect of salicylates was not on the kidney alone.

Longenecker, Fricke and King (85) studied the effects of many barbituric acid derivatives, hypnotics, and antipyretics on the excretion of ascorbic acid in the urine of albino rats. Among the more active compounds assayed were chloretone, paraldehyde, sodium phenobarbital, and calcium ipral. The administration of 20 milligrams of chloretone daily caused a steady rise in the urinary ascorbic acid output which reached a maximum of 20 to 30 milligrams on the twelfth to fourteenth day of feeding and remained at that level for a three months test period. Both chemical and biological determinations were made on the urine and close agreement was found. No harmful effects were noted in the experimental animals. The authors con-

cluded that the functional relationships of the active compounds studied pointed toward a close connection between vitamin C and the metabolism of nerve tissue since increased synthesis of ascorbic acid constitutes a fairly rapid and continued response of the rat to many nerve depressants. They suggested that possibly the accelerated ascorbic acid synthesis is a protective mechanism available to the animal against foreign toxic substances. Prior to the experimental work cited above the known functions of chloretone were limited to those of a rather inferior anesthetic and a preservative. Hamilton (86) contrary to most workers was of the opinion that chloretone had no equal as a general anesthetic because of its long continued action and non-interference with the circulatory system. Polak (87) was not in agreement with this and stated that chloretone injures the blood circulation by paralyzing heart muscles and the cardiac centers.

Draining tissues

Many compounds in addition to sulfur and salicylates (64)(84) have been found to lower the ascorbic acid in body tissues. Samuels and coworkers (88) reported a depletion of body reserves of ascorbic acid by the administration of acetylsalicylic acid as was indicated by a definite increase in the excretion during the administration of the drug, followed by a subnormal excretion when feeding of the drug was stopped. Svirbely (65) reported that the parallel decrease in ascorbic acid of the liver and the gut when bromobenzene and iodoacetic acid were fed indicates a decreased synthesis rather than an increased requirement of the vitamin for detoxication. It was assumed that the gut is the site of synthesis, and that these halogenated compounds interfere with the functions of the intestinal mucosa.

Drake and associates (89) using albino rats on a vitamin C free diet found that dilantin (sodium diphenyl hydantoinate) decreased the concentration

of ascorbic acid in the tissues by increasing the urinary excretion of the vitamin. Doses of 500 milligrams were given. Decreased ascorbic acid concentration in the liver, brain, muscles, adrenals and blood was observed. Dainow (90) found that the administration of certain sulfur derivatives of benzene such as uliron, septoplix, and rodilone caused a decrease in the ascorbic acid content of the testicles, liver and brain of the guinea pig.

Bersin and associates (91) produced ascorbic acid deficiency in rabbits by intravenous injections of silver chloride. Rabbits three or four months of age were used and an estimation was made of the ascorbic acid content of blood, liver, intestines and kidneys. Ascorbic acid values were lowered in every case in animals receiving silver chloride, but were maintained about normal in those animals receiving ascorbic acid as well.

Other factors

There are many indications that the ascorbic acid level in the blood is closely associated with the metabolic rate. Studies of the effects of insulin and adrenalin have shown this. Daoud and others (32) found that the increased metabolic rate after adrenalin administration, without any increased synthesis caused a marked drop in the urinary output of the vitamin. On the other hand when the metabolism was lowered by starvation for 24 hours less ascorbic acid was used as was indicated by the increased urinary output.

The work of Ralli and Sherry (33) on dogs did not support this theory. Insulin injections lowered the plasma level of ascorbic acid and decreased the excretion. The influence of nicotine on vitamin C metabolism was studied by Strauss and Scheer (92). The subjects, saturated with ascorbic acid, were given a test dose of 200 milligrams and the effect of smoking on the urinary excretion of ascorbic acid was determined. The marked reduction in urinary ascorbic acid following smoking was thought to be related to the endocrine

mechanisms (thyroid and adrenal) and a higher level of general oxidative processes. Wilczek (93) found that guinea pigs killed by various methods showed an increase in blood ascorbic acid in proportion to the length of the period of agony. He attributed this to the liberation of adrenalin.

Vitamin Interrelationships

Sure, Theis, and Harrelson (94) made a study of the effects of certain vitamin deficiencies on the ascorbic acid concentration in the tissues of rats. In vitamin B_1 deficiency there was a pronounced reduction of ascorbic acid in the lungs, liver, kidneys and thymus. Riboflavin deficiency caused greater losses than other avitaminosis; losses of 41 to 45 per cent of ascorbic acid were found in the lungs, kidneys and liver and an 85 per cent reduction in the thymus ascorbic acid was noted. The studies on the relationship of vitamin A deficiency to ascorbic acid metabolism were too limited to be significant. Phillips and associates (95) in their studies on calves, however, reported that the blood plasma ascorbic acid declined to a relatively constant low level with the onset of vitamin A deficiency and that the urinary output of ascorbic acid per day was greatly reduced. Hansard and Sutton (96) using rats for experimental animals reported that a restriction of the vitamin A intake caused a marked decrease in the blood vitamin C. Kimble and Gordon (97) in studying vitamin A deficiency in humans by blood levels and biophotometer performances found several cases where the administration of vitamin A alone did not improve conditions but when ascorbic acid was given in addition satisfactory improvement was noted.

Ascorbic Acid Metabolism in Dairy Animals

Synthesis of ascorbic acid

As early as 1926 Thurston and coworkers (98) reported results which indicated that dairy animals were capable of synthesizing ascorbic acid and

that the ascorbic acid content of the ration was of no significance. Animals fed a vitamin C free ration for 365 days did not show any signs of scurvy. To eliminate the possibility of storage of the vitamin a calf was obtained from a cow that had been fed a vitamin C free ration for three months before calving. This calf was raised to maturity on this same vitamin C deficient ration, and she in turn produced a normal calf. In a later experiment the same workers (99) showed that the liver extract of calves on a vitamin C free ration was equal to that of control animals in anti-scorbutic potency. The work of Hjarre and Lilleengen (100) was not in agreement with these findings. These workers found waxy muscle degeneration in vitamin C deficient calves. One calf showed gross macroscopic signs of muscle degeneration and two others showed degenerative changes in the muscle and scorbutic signs in the teeth upon microscopic investigation. Experiments performed by Knight and associates (101) demonstrated the rapid destruction of ascorbic acid fed to dairy cows. A cow was fed 2,000,000 International units of vitamin C with no increase in the ascorbic acid values of the blood plasma nor of the milk as compared with those values obtained when the cow was on the standard unsupplemented ration. Only a slight increase in urinary output was noted. Similar results were obtained when 100 grams of ascorbic acid were placed directly in the rumen through a fistula opening. The rapid destruction of the vitamin added to rumen contents in vitro at 39° - 42° C. was also shown. There is some experimental evidence that certain roughages may affect ascorbic acid metabolism in ruminants. Riddell and Whitnah (102) reported that the average vitamin C content of the blood more than doubled within 12 hours after cows were changed from a winter ration to all the green rye they would consume. Twelve hours later the blood level reverted to normal. The average output of ascorbic acid in the urine

was increased to over five times normal within 60 hours after the green rye was first fed. Rasmussen and coworkers (103) reported that succulent pastures were effective in increasing the ascorbic acid content of milk of various species.

In their work with goats Richmond and others (104) found that the level of ascorbic acid in the blood was not significantly affected by the change from pasture to a vitamin C free diet.

Ascorbic acid therapy

Although it has been definitely shown that dairy animals are capable of synthesizing sufficient ascorbic acid to maintain the animal in a healthy condition it would appear from the recent work of Phillips (35)(36) and others that in certain individual animals there is a need for additional amounts of the vitamin for reproduction. Phillips and associates (35) made subcutaneous injections of ascorbic acid in bulls that showed slowness of breeding, general sexual indifference, and a low potency rating. These workers reported success in 80 per cent of the cases treated. They were of the opinion that the ascorbic acid content of fresh semen and freshly drawn blood plasma is indicative of the potency or impotency of the bull. Studies of the relationship of ascorbic acid to reproduction in the cow were also made by Phillips and associates (36). Cows that were regarded as "hard to settle" but whose reproductive tract appeared normal responded in a large number of cases to ascorbic acid therapy. These workers believe that the ascorbic acid concentration of blood plasma increases for a short period during the height of estrum in most cows with regular and consistent breeding history. "Hard to settle" cows fail to show this peak.

Summary of Review of Literature

The nutritional importance of ascorbic acid has been widely recognized and has provided the stimulus for widespread investigation and experimentation with this nutrient. Since the guinea pig, man, monkey and other primates are limited to their diet as a source of this vitamin most of the experimental work has been done with them. In other animals the capacity to synthesize this vitamin and the infrequency with which deficiencies have been observed have limited the knowledge of ascorbic acid metabolism in these animals.

Most chemical methods for the determination of ascorbic acid are based on the reduction of the dye 2, 6-dichlorophenol indophenol which was first used by Tillmans and associates. Mindlin and Butler adapted this method to the determination of plasma ascorbic acid and used the photoelectric colorimeter to measure the amount of dye reduced. Due to the many hazards of a chemical determination of this nature many modifications of the original method and many new methods have been devised. The use of mercuric acetate to precipitate reducing substances other than ascorbic acid was suggested but most workers did not find this procedure advantageous.

The ease with which ascorbic acid is oxidized makes it essential that blood samples be properly protected after being drawn. It was shown that the intact red cells protected ascorbic acid from oxidation, but the vitamin was readily oxidized in hemolyzed samples. The use of potassium cyanide for protecting ascorbic acid in blood samples was suggested but is not generally accepted.

The two major physiological functions of ascorbic acid are: the regulation of the colloidal condition of intercellular substances and a respiratory function of giving off and taking up hydrogen atoms. This vitamin is also important in the defense mechanisms of the body and in reproduction.

Experiments set up to produce scurvy in man and to determine minimum requirements showed that the tissues hold reserve supplies sufficient to prevent any macroscopic signs of the vitamin deficiency for several months after the individual has been put on a vitamin C free diet.

The concentration of ascorbic acid in the various tissues of many animals was studied and although normal tissues from different animals did not fall in exactly the same sequence the general order of decreasing concentration was pituitary body, corpus luteum, adrenal cortex, young thymus, liver, brain, testes, ovaries, spleen, thyroid, muscle, spinal fluid and blood.

Many constituents of the body were shown to protect ascorbic acid from destruction. Glutathione is probably the most effective of these constituents.

Mannose, glucose, and galactose were shown by some workers to be precursors of ascorbic acid in "in vitro" experiments. The role of manganese in ascorbic acid synthesis is a controversial subject. Recent work showed that certain chemically unrelated compounds including carvone and chloretone, stimulated synthesis and increased urinary output in those animals capable of ascorbic acid synthesis while other compounds including salicylates and dilantin caused a draining of the tissues of this vitamin. Some evidence has been presented that there is an interrelationship between ascorbic acid and vitamin A and some members of the vitamin B complex.

The capacity of the dairy cow to synthesize ascorbic acid and fortify milk with this nutrient which is so essential to man has created considerable interest in this respect. Recent investigations have also been concerned with the role of this vitamin in maintaining normal reproduction in dairy animals.

OBJECT

The object of this experiment was to determine the plasma ascorbic acid level of dairy animals of various ages and to study the normal variation in the plasma ascorbic acid through the day and from day to day. In addition a study was made of some of the factors affecting the plasma ascorbic acid level.

EXPERIMENTAL PROCEDURE

Animals Used

The animals used in this experiment were from the Michigan State College Experimental herd and included representatives of the Brown Swiss, Guernsey, Holstein and Jersey breeds. These animals varied in age from calves a few days of age to mature cows. Cows in various stages of lactation and gestation were included. Ten of the animals used were on rations that were adapted to a study of the effect of the ration upon the plasma ascorbic level, while the remaining animals were on other feeding experiments.

Bleeding Procedure

The animls were bled at approximately the same time each day. The regular bleeding time was 6:30 a. m. to 7:30 a. m. except when the animals were bled several times each day. Immediately after the blood samples were drawn they were placed on ice and protected from light until they were taken to the laboratory. The frequency of bleeding depended somewhat on the type of experiment but in most cases the animals were bled once each week.

Determination of Plasma Ascorbic Acid

The procedure used for analysis of the plasma ascorbic acid in this study was a slight modification of the macromethod of Mindlin and Butler (9). The addition of a five per cent solution of potassium cyanide to the blood

sample for protection of the ascorbic acid as outlined in the above procedure was omitted since the work of many investigators indicated that this procedure had no advantage. Twenty-five ml. of venous blood were collected in a pyrex tube containing six to eight drops of a 20 per cent solution of potassium oxalate. In mixing the anticoagulant with the blood the samples were shaken gently as a guard against breaking of the red cells. The samples were placed on ice immediately, protected from light, taken to the laboratory, and centrifuged within one hour after being collected. In the early part of the experiment when determinations could not be made immediately the plasma was held in the ice box up to 12 hours. Later it was found more desirable to prepare the filtrate immediately after centrifuging the samples and then if necessary hold the filtrate in the ice box up to 12 hours. The filtrate was prepared by pipetting two ml. of plasma into a test tube containing two ml. of distilled water. Four ml. of a five per cent solution of metaphosphoric acid were added and mixed by shaking gently. The mixture was filtered through a number two Whatman filter paper and the filtrate collected. At this point it was necessary to alter the macro procedure slightly because the only apparatus available was for micro determinations. One ml. of the filtrate was added to one ml. of the dye solution in the cell and the amount of dye reduced was measured colorimetrically. In calculating the results the original formula was used and the results multiplied by four to compensate for using only one ml. of filtrate.

The indophenol-acetate solution was made up as described in the procedure except it was diluted so that the blank analysis gave a galvanometer reading of 70. This weaker dye solution gave a wider range between the blank reading and the sample reading and reduced the error in the results. Dupli-

cate readings were made on each filtrate and an average of the two used in the calculation. The K value which was used in the calculations was checked at various intervals by the use of standard solutions made up in 2.5 per cent metaphosphoric acid solution from crystalline ascorbic acid.

RESULTS

Normal Variations in Blood Plasma Ascorbic Acid of Dairy Animals of Various Ages

Calves

The plasma ascorbic acid values found for calves up to ten weeks of age are shown in table I. Blood samples were taken the same day of each week in most cases and at approximately the same time each day. When the calves were bled twice in one week an average of the two values obtained was used. All these calves were fed a whole milk ration.

Table I. Blood Plasma Ascorbic Acid in Calves up to Ten Weeks of Age

	Milligrams Ascorbic Acid per 100 ml. Blood Plasma									
Animal No.	Age in Weeks									
	1	2	3	4	5	6	7	8	9	10
C-460				.138	.148	.170	.092	.306	.114	.206
C-461	.047	.186	.138	.245	.123	.118	.261	.213		
C-462		.431	.263	.213	.415	.350	.276	.505	.294	.472
C-463	.447	.283	.533	.502		.399		.126		
C-464		.256	.332	.343	.209	.278	.381			
C-465				.343	.431	.544	.472	.420	.440	.549

The data showed that there was a wide variation in the plasma ascorbic acid values of calves fed the same ration. These values were obtained during the fall and winter months.

Heifers

The plasma ascorbic acid level for heifers between 12 and 36 months of age was studied. All these animals were open except A-29. These animals were bled several times during each month and an average of the values obtained for each month is shown in table II.

Table II. Blood Plasma Ascorbic Acid Values of Heifers 12 to 36 Months of Age

Animal No.	Milligrams Ascorbic Acid per 100 ml. Plasma						
	January	February	March	April	May	June	July
A-29	.739	.680	.527		.615		
A-31	.537	.575	.479				
A-33	.632	.473	.447		.533	.564	
A-34					.675	.474	
A-35	.458	.361	.263		.477	.444	
16						.455	.389

In most cases the level of plasma ascorbic acid for these heifers was more than 100 per cent higher than the values obtained for the calves shown in table I.

Cows

The blood plasma ascorbic acid values for six milking Holstein cows are shown in table III. These blood samples were taken at irregular intervals, usually in connection with other experiments.

Table III. Blood Plasma Ascorbic Acid Values of Milking Cows

Animal No.	Milligrams Ascorbic Acid per 100 ml. Plasma — Sample No. 1	2	3	4	5	6	7	8	9	10
	1-17*	2-14	2-18	2-24	3-5	3-12	3-19	3-26		
269	.294	.549	.520	.384	.277	.384	.434	.419		
	1-17	2-14	2-18	2-25	3-5	3-8				
285	.225	.480	.534	.370	.316	.384				
	1-17	2-8	2-14	2-18	2-24	3-5	3-8			
289	.314	.497	.480	.606	.384	.200	.472			
	5-6	5-10	5-12	5-14	5-19	5-21	5-23	5-26		
290	.292	.252	.583	.538	.583	.581	.373	.569		
	1-17	2-12	3-31	5-20	7-28					
A-26	.467	.578	.507	.581	.583					
	1-9	1-10	1-12	1-17	1-20	1-24	2-24	5-16	7-28	
A-27	.735	.537	.608	.544	.480	.668	.578	.566	.606	

*Date

These data show variations of more than 100 per cent between milking cows, and also wide variations by the individual cows. The average value obtained for milking cows was somewhat lower than that found for heifers.

Checking the Method of Analysis

The wide range of plasma ascorbic acid values obtained for individual animals in the previous studies indicated a normal daily variation. To eliminate any possibility of these observed variations being due to error in the determinations, the method of analysis was checked for variations due to such factors as fading of the dye and possible changes occurring in the colorimeter over the period studied.

Five cows were bled at three hour intervals for a period of 24 hours. A large sample of blood was drawn at the first bleeding. Ten ml. of filtrate was prepared from this first sample and an additional ten ml. of plasma retained. The filtrate and the plasma were held in the ice box and used for checks. Each time that a reading was made on the samples taken at the various intervals, readings were also made on a filtrate prepared from the plasma held from the first sample for a check and on the filtrate held for check. Any variations shown in values for the filtrate held for check were regarded as slight, unavoidable errors in reading the galvanometer except in the case of the 4:00 a.m. readings on the samples of cows A-22 and 264. These two high values were regarded as errors in the determination rather than changes occurring in the filtrate on standing.

The data indicated that there was a significant loss of ascorbic acid in plasma held for twelve hours, that filtrates for ascorbic acid analysis should be prepared immediately after the plasma is obtained, and that these filtrates may be held for as long as 24 hours without significant destruction of the vitamin.

The data shown in table IV indicate that the variations in the values obtained for plasma ascorbic acid at three hour intervals over a period of 24 hours were due to actual variation in the ascorbic acid level and that there were only slight variations due to errors in the method of determination.

Daily Variations in Blood Plasma Ascorbic Acid

The previous experiments in which the animals were bled once or twice each week and the plasma ascorbic acid determined showed that there was a wide variation in the values obtained. An experiment was set up to determine how much the plasma ascorbic acid varied in normal animals from day to

Table IV. Variations in Blood Plasma Ascorbic Acid of Dairy Cattle at three Hour Intervals and the Stability of Ascorbic Acid in Plasma and in Plasma Filtrate

			1:00 p.m.	4:00 p.m.	7:00 p.m.	10:00 p.m.	1:00 a.m.	4:00 a.m.	7:00 a.m.	10:00 a.m.	1:00 p.m.	4:00 p.m.
A-22	Filtrate	I*		0.402	0.370	0.285	0.441	0.541	0.378	0.444	0.547	0.460
3/24	"	II**			0.389	0.305		0.345	0.244			
	"	III***		0.402	0.402	0.402	0.402	0.476	0.391	0.431	0.431	0.427
264	Filtrate	I		0.517	0.529	0.338	0.454	0.476	0.444	0.496	0.547	0.447
3/24	"	II			0.485	0.485		0.470	0.444			0.354
	"	III		0.517	0.529	0.518	0.466	0.541	0.509	0.509	0.509	0.557
237	Filtrate	I		0.701	0.761	0.591	0.529	0.623	0.604	0.685	0.759	0.608
3/24	"	II			0.761	0.701		0.667	0.685			
	"	III		0.701	0.701	0.701	0.701	0.635	0.597	0.597	0.697	0.733
266	Filtrate	I	0.395	0.441	0.612	0.520	0.398	0.483	0.538	0.496	0.508	
3/31	"	III	0.419	0.399	0.399	0.416	0.416	0.416	0.416	0.386	0.386	
A-25	Filtrate	I	0.478	0.513	0.772	0.632	0.615	0.609	0.615	0.508	0.477	
	"	III	0.507	0.501	0.501	0.508	0.508	0.508	0.508	0.465	0.465	

*Filtrate prepared from plasma taken at three hour intervals.

**Filtrate prepared at three hour intervals from plasma of first sample.

***Filtrate prepared at start of experiment from plasma of first sample.

day. The animals used were lactating Holstein cows which were either open or pregnant. The animals were bled at approximately the same hour each day, 7:00 a.m., and the blood sample protected so as to prevent any variation in results due to the destruction of ascorbic acid. Five cows were used in the first group and samples were taken each day for ten days. The second group was made up of three cows, which were bled daily for eight days.

The data presented in table V show that there was a wide variation in the values obtained for each animal but since all of the animals did not vary in the same direction at any particular time, no indication of the cause of these variations was found. These data indicate that the normal daily variation in the plasma ascorbic acid of milking cows is more than 100 per cent.

Variations in Blood Plasma Ascorbic Acid at Three Hour Intervals

This experiment was set up to study the influence of the time of bleeding on the plasma ascorbic acid and to determine if a significant difference occurred at various hours during the day.

One Brown Swiss, two Jersey, and 11 Holstein cows were used for this study. Each of these animals was bled at three hour intervals for a period of 24 hours. The samples were handled in the usual manner, centrifuged within two hours after being drawn and the plasma held in the ice box until the filtrates were prepared.

The data in table VI show that there was a wide variation in the plasma ascorbic acid values of individual animals during the day. There was also a wide variation between animals. The time at which the maximum and minimum values occurred did not appear at the same hour of the day in all animals.

Table V. Daily Variation in Blood Plasma Ascorbic Acid of Lactating Holstein Cows

Animal No.	Milligrams Ascorbic Acid per 100 ml. Plasma — Days									
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
A-6	.328	.425	.454	.293	.225	.284	.225	.166	.248	.255
A-23	.592	.592	.512	.367	.475	.440	.284	.188	.218	.156
269	.520	.534	.396	.323	.334	.384	.384	.240	.323	.278
285	.534	.549	.396	.345	.353	.370	.298	.370	.293	.367
289	.606	.498	.308	.396	.298	.355	.384	.188	.323	.308
A-18	.560	.585	.591	.557	.731	.703	.501	.560		
267	.402	.428	.325	.421	.552	.528	.478	.417		
D-9	.357	.325	.305	.348	.533	.420	.436	.257		

This made it impossible to correlate these variations with any known factor such as feeding time, resting time, or milking time. These data indicate that individual animals normally vary as much as 100 per cent in their plasma ascorbic acid level and that these variations do not occur at regular intervals.

The variations in plasma ascorbic acid of one Jersey cow, 66, and one Holstein heifer, C-424, were studied over a period of 48 hours. In this experiment the length of the bleeding interval was increased to six hours. It is shown in table VII that minimum and maximum plasma ascorbic acid values do not occur at regular intervals but may occur at any time during the day.

Table VI. Variations in Blood Plasma Ascorbic Acid of Dairy Animals at Three Hour Intervals

	Milligrams Ascorbic Acid per 100 ml. Plasma													
	76	264	267	A-18	A-21	A-26	78	239	266	A-14	A-22	A-27	C-424	D-14
7:00 a.m.	.375	.433	.532	.621	.461	.669								
10:00 a.m.	.244	.390	.461	.375	.375	.601								
1:00 p.m.	.154	.229	.433	.375	.532	.656	.502	.502	.569	.350	.308	.766	.488	.279
4:00 p.m.	.093	.347	.461	.723	.532	.621	.515	.569	.569	.364	.364	.701	.433	.293
7:00 p.m.	.169	.288	.433	.390	.447	.656	.529	.502	.583	.364	.308	.688	.364	.258
10:00 p.m.	.461	.266	.504	.433	.504	.587	.433	.433	.502	.430	.286	.623	.293	.236
1:00 a.m.	.139	.214	.518	.504	.482	.601	.364	.474	.488	.364	.293	.609	.236	.258
4:00 a.m.	.229	.229	.461	.518	.504	.710	.364	.433	.468	.364	.350	.779	.308	.329
7:00 a.m.							.364	.420	.468	.308	.329	.636	.350	.279
10:00 a.m.							.433	.433	.556	.329	.336	.569	.350	.308
1:00 p.m.							.364	.433	.468	.293	.236	.701	.293	.364

Table VII. Variations in Plasma Ascorbic Acid over a 48-hour Period

Cow No.	Day of Experiment	Milligrams Ascorbic Acid per 100 ml. Plasma 7:00 a.m.	1:00 p.m.	7:00 p.m.	1:00 a.m.
	first	.080	.284	.144	.144
66	second	.236	.144	.208	.250
	third	.215			
	first	.326	.298	.222	.264
C-424	second	.277	.291	.400	.208
	third	.229			

Variations in Plasma Ascorbic Acid of Blood Samples that Did not Show Hemolysis

In the previous experiments there were a few plasma samples that showed evidence of hemolysis. The experiment was repeated and all samples were observed for hemolysis. The bleeding technique was improved and there were no signs of hemolysis of any of the samples in this experiment. All filtrates were prepared and placed in the ice box within one to two hours after the samples were taken. All ascorbic acid determinations were made at the end of the 24-hour period. The data in table VIII indicate that the low plasma ascorbic acid values obtained at various hours during the day in the previous experiments were not due to destruction of ascorbic acid caused by hemolysis of the samples.

The Effect of Various Feeds on the Blood Plasma Ascorbic Acid Level

Milk low in ascorbic acid

One Guernsey and three Holstein calves were used in this experiment. Holstein calves C-466 and C-468 were started on this experiment when they were about one week of age. Guernsey calf C-464 and Holstein calf C-460

Table VIII. Variations in Plasma Ascorbic Acid of Blood Samples that Did not Show Hemolysis

	Ascorbic Acid per 100 ml. Plasma			
	66	C-405	A-26	A-27
	mg.	mg.	mg.	mg.
11:00 a.m.	.458	.385	.717	.665
2:00 p.m.	.372	.354	.636	.595
5:00 p.m.	.372	.310	.654	.683
8:00 p.m.	.241	.329	.619	.536
11:00 p.m.	.247	.285	.660	.700
2:00 a.m.	.378	.285	.803	.717
5:00 a.m.	.403	.304	.595	.494
8:00 a.m.	.323	.348	.512	.482
11:00 a.m.	.171	.266	.397	.578

were six weeks and sixteen weeks of age respectively, when the experiment was started. The milk fed to these calves contained approximately one milligram ascorbic acid per liter. The data shown in table IX indicate

Table IX. Plasma Ascorbic Acid in Calves on Milk Low in Ascorbic Acid

	Before Feeding Milk Low in Ascorbic Acid	After Feeding Milk Low in Ascorbic Acid — Week						
		1st	2nd	3rd	4th	5th	6th	7th
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
C-460	.280	.439	.215	.240	.298	.172	.251	
C-464	.381	.551	.407	.240	.388	.195	.316	.426
C-466	.444	.285	.284	.328	.211	.076	.203	.218
C-468	.452	.368	.475	.520	.440	.331	.326	.358

that calves fed milk low in ascorbic acid maintain a blood plasma ascorbic acid level similar to that observed for calves of the same age which were fed a normal ration (table I).

Concentrates

An eight months old Holstein heifer, 413, and a 12 months old Guernsey heifer, 23, were used in this trial. Both heifers were put on a ration of ten pounds of alfalfa hay and four pounds of corn for five weeks and the level of the plasma ascorbic acid determined. Both animals were changed to a corn and beet pulp ration in order to study the effect of feeding concentrates on the plasma ascorbic acid level. Since heifer 23 did not eat the beet pulp well she was changed to a ration of four pounds of corn and four pounds of soybean oil meal at the end of one week. After two weeks on the corn and beet pulp ration heifer 413 was changed to ten pounds of beet pulp alone. The heifers remained on these rations for more than four months. Blood samples were taken once each week during most of this time and the level of plasma ascorbic acid determined. After studying the effect of these rations on the level of ascorbic acid in the plasma it was apparent that there was no significant change as may be noted in Fig. I. The next concentrates studied were corn and tankage fed to heifer 23 and meat scraps and beet pulp fed to heifer 413. The heifers were on these rations for about one month. There was no significant change in the plasma ascorbic acid level in the case of heifer 23. Heifer 413 showed a trend toward a lower level of plasma ascorbic acid at the end of this experiment. At this time, however, she was beginning to show symptoms of a vitamin A deficiency which may account for the results. The data obtained indicate that the feeds used in these experiments had no significant effect on the level of plasma ascorbic acid. Since there were wide variations in the values obtained for these heifers while on each of the experiments no in-

dividual value was considered significant but the level over a period of several weeks was used in evaluating the results.

Alfalfa hay

A five year old Holstein cow, A-19, and a two year old Holstein heifer, C-424, were used to study the effect of feeding alfalfa hay upon the plasma ascorbic acid level. The heifer had been fed a basal ration and the cow had been on alfalfa hay and corn for several months before the experiment was started. Several blood samples were taken while the animals were on these rations to determine the level of plasma ascorbic acid. The rations were then reversed and the effect on plasma ascorbic acid noted. While A-19 was on the basal ration she developed a vitamin A deficiency. Cod liver oil was added to her ration to supply vitamin A.

The plasma ascorbic acid was maintained at a higher level when these two animals were fed alfalfa hay than when they were fed the basal ration with no roughage.

Blood Plasma Ascorbic Acid of Vitamin A Deficient Calves and Heifers

Calves and heifers that were on an experiment to determine the vitamin A requirements for growth were used in this study. These animals were fed various levels of carotene as is indicated in table X.

The values obtained for these animals were slightly lower than the average values reported for normal dairy animals. However, no extremely low values were found except for heifer C-439 which showed severe symptoms of vitamin A deficiency when this study was started. This animal died of vitamin A deficiency two weeks later. The four year old heifer, C-405, which had received 14 micrograms of carotene per pound of body weight for a considerable length of time showed a plasma ascorbic acid level that was within the normal range reported in the literature. However, her average

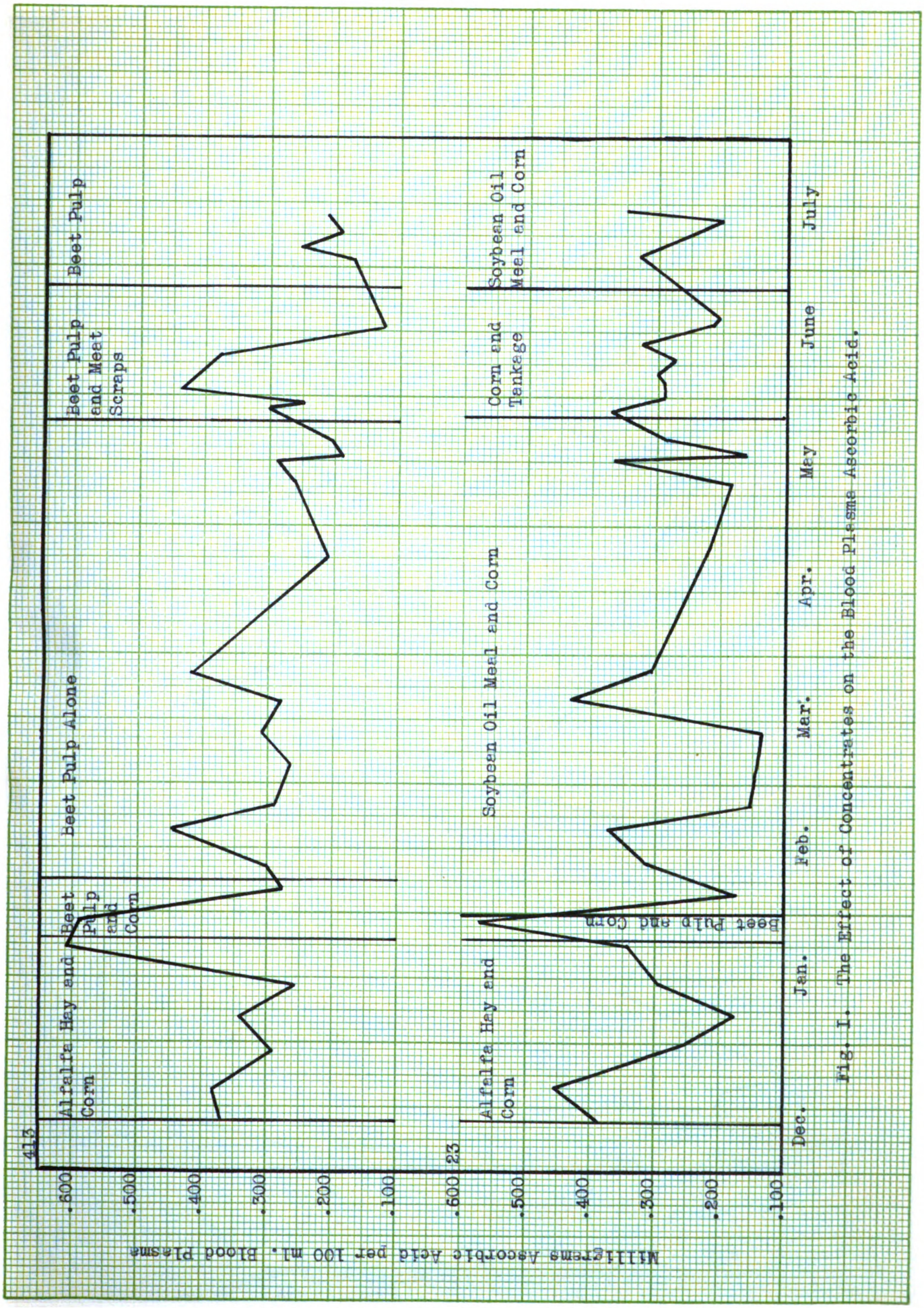

Fig. I. The Effect of Concentrates on the Blood Plasma Ascorbic Acid.

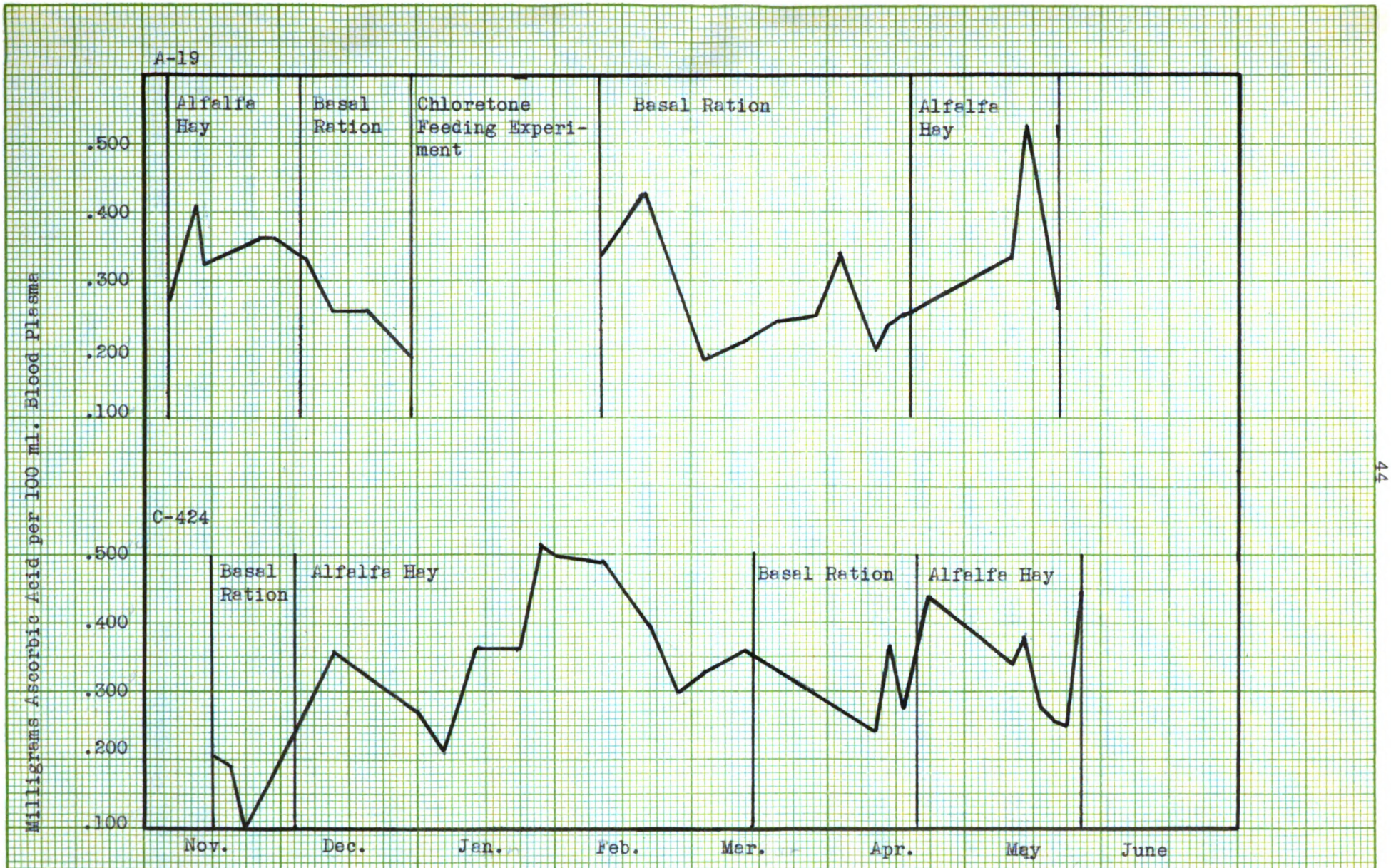

Fig. II. The Effect of Feeding a Basal Ration on the Blood Plasma Ascorbic Acid Level.

Table X. Milligrams Ascorbic Acid per 100 ml. Blood Plasma of Vitamin A Deficient Calves and Heifers

	C-405	C-439	C-450	C-453	C-456	C-457
	Micrograms of carotene per pound body weight					
Date	14	None	28	None	35	35
	Mg Per cent	Mg Per cent	Mg Per cent	Mg Per cent	Mg Per cent	Mg Per cent
4/7/41		0.068				
4/8/41	0.202	0.027	0.241	0.228	0.267	0.228
4/10/41	0.201	0.102	0.298	0.208	0.292	0.208
4/15/41	0.305	0.014	0.292	0.324	0.343	0.273
4/17/41	0.277	0.088	0.284	0.206	0.303	0.200
4/22/41	0.172		0.338	0.232	0.212	0.179
4/29/41	0.279		0.362	0.227	0.343	0.279
5/6/41			0.318	0.292	0.219	0.292
5/13/41	0.319		0.319	0.240	0.312	0.306
5/15/41	0.226		0.219	0.278	0.226	0.200

plasma ascorbic acid value was slightly below the normal for the Holstein breed.

It was observed that these animals on a low vitamin A ration did not show as wide a variation in plasma ascorbic acid as was found in animals on a normal ration. The absence of high maximum values was very noticeable in these animals.

The Effect of Feeding Dill Seed Oil on Blood Plasma Ascorbic Acid

The work of Longenecker and others (83) and that of Ritz and coworkers (84) suggested the possibility that dill seed oil which is about 60 per cent carvone might increase ascorbic acid synthesis. This experiment was set up

to determine the effect of feeding this oil on ascorbic acid synthesis in dairy animals. Due to the strong odor and taste of the oil the cows and heifers would not eat grain after dill seed oil was added to it. The next method of feeding the oil was to spray it on the hay. Two heifers, 23 and 413, were fed ten pounds of alfalfa hay daily that had been sprayed with five ml. and later 7.5 ml. dill seed oil. After feeding the dill seed oil at this rate for 18 days no significant effect on the plasma ascorbic acid was noted. The dose was increased to ten ml. per day and fed in a gelatin capsule. The oil was diluted with an equal volume of butter to reduce the irritation of the digestive tract. After 13 days the dose was increased to 20 ml. of dill seed oil daily and feeding was continued at this level for 16 days.

The data presented in table XI show a slight increase in plasma ascorbic acid during the period of dill seed oil feeding but in view of the normal daily variations in plasma ascorbic acid this increase cannot be regarded as being significant.

Table XI. The Effect of Feeding Dill Seed Oil on Blood Plasma Ascorbic Acid

		Ascorbic Acid per 100 ml. of Plasma					
			Dill Seed Oil				
Animal No.		Before Feeding	5 ml.	7.5 ml.	10 ml.	20 ml.	After Feeding
		mg.	mg.	mg.	mg.	mg.	mg.
23	1st wk.	.236	.337	.413	.452	.329	.458
	2nd wk.	.294		.371	.214	.386	.279
413	1st wk.	.460	.245	.494	.383	.291	.383
	2nd wk.	.186	.	.272	.272	.373	.293

The Effect of Feeding Chloretone on the Blood Plasma Ascorbic Acid of Dairy Cattle

Since the work of Longenecker and associates (85) showed that chloretone was the most potent compound that they observed for increasing urinary excretion of ascorbic acid by rats, it was chosen for the second feeding experiment. Seven Holstein and two Jersey cows were used in the first group. These animals were on various rations which consisted chiefly of alfalfa hay with various supplements.

The methods of feeding and doses of chloretone were varied from a single 40-gram dose given in a gelatin capsule to a five-gram dose fed daily with the grain for a period of 21 days. Blood samples were taken two or three times each week and the plasma ascorbic acid values obtained were averaged for the week. The "peak" which was the highest single value obtained was noted and is shown in table XII.

Table XII. The Effect of Feeding Chloretone on the Blood Plasma Ascorbic Acid of Dairy Cows

				Ascorbic Acid per 100 ml. of Plasma					
Cow	Wt. of Cow	Daily Dose	Days Fed	Ave. of 10 days Before	After Initial Feeding of Chloretone 1st wk.	2nd wk.	3rd wk.	4th wk.	Peak
No.	lbs.	gm.		mg.	mg.	mg.	mg.	mg.	mg.
269	1200	40	1	.426	.526	.612	.418	.416	.369
A-19	1150	20	4	.214	.637	.555	.472	.336	1.032
D-5	1130	20	5	.390	.575	.905	.423	.156	.920
A-21	1170	10	10	.473	.923	.971	.632	.441	.962
66	740	10	10	.423	.389	.688	.369	.389	.859
264	1270	10	7	.538	.427	.331	.393	-	.557
264*	1270	5	15	.362	.534	.878	.503	-	1.095
267	1130	5	15	.454	.641	.622	.693	.382	.808
285	1300	5	18	.350	.520	.607	.643	.513	.719
289	1320	5	21	.336	.456	.526	.595	.505	.770

*11 days after last dose in first experiment

The peak occurred within 12 days after the initial dose when 10 grams or more of chloretone were fed in a single dose. However, the "peak" was not reached in less than two or three weeks when daily doses of five grams were fed. Regardless of the ration fed, stage of lactation or gestation, the feeding of chloretone resulted in a significant increase in the plasma ascorbic acid as shown in table XII. The plasma ascorbic acid returned to the pre-feeding level within two weeks following the last feeding of chloretone. Doses of ten grams or more per day caused a drop in milk production in certain individual cows and in some cases this was accompanied by an anesthetic effect. However, all animals returned to normal when treatment was stopped. The most extreme case was that of cow 269 which received a single 40-gram dose. During the first 24 hours following this dose she went off feed, could not walk without falling and showed a marked decrease in milk production. During the second 24 hours following the dose she was wobbly but started eating and began to return to normal milk production. One week after the dose was given this cow had apparently returned to normal in every respect except that her plasma ascorbic acid remained at a high level.

The response of cow 264 to the first chloretone feeding period was interesting. As is shown in table XII she was fed ten grams of chloretone daily for seven days. After five days she went off feed, decreased in milk production and her plasma ascorbic acid level decreased. Eleven days after discontinuing chloretone feeding at the ten-gram level this cow had returned to normal. At this time she was again started on a chloretone feeding experiment. This time the level of feeding was reduced to five grams per day. During the second period this cow did not show any ill effects and she showed an increase in her plasma ascorbic acid level. These data suggest the possibility that this cow was deficient in ascorbic acid in her tissues at the

time the first feeding period was started. By the time the second feeding period was started this cow's tissues may have become saturated with ascorbic acid and her plasma ascorbic acid level increased due to the feeding of chloretone.

Two heifers were used to determine the minimum dose of chloretone that would produce an increase in the plasma ascorbic acid. Table XIII shows the effect of feeding one five-gram and three five-gram doses of chloretone.

Table XIII. The Effect of Feeding Small Doses of Chloretone on Blood Plasma Ascorbic Acid

Heifer No.		Ascorbic Acid per 100 ml. of Plasma					
		Ave. of 10 days before	After Initial Feeding of Chloretone				
			1st wk.	2nd wk.	3rd wk.	4th wk.	Peak
A-33	One 5-gram dose	.564	.618	.578			.745
	Three 5-gram doses	.586	.737	.394	.479	.501	.922
A-34	One 5-gram dose	.474	.612	.483			.639
	Three 5-gram doses	.495	.634	.529	.442	.441	.719

The small increases in plasma ascorbic acid after feeding one five-gram dose of chloretone to each of these heifers indicated that this one dose may have raised the plasma ascorbic acid level but in view of the wide normal daily variations the significance of these increases is questionable. The increases shown as a result of feeding three five-gram doses appeared significant.

Chloretone was shown to be effective in increasing the plasma ascorbic acid level of one Jersey and two Holstein bulls. It was observed in this experiment that the plasma ascorbic acid level dropped below the prefeeding level in two of the bulls when chloretone feeding was discontinued.

Table XIV. The Effect of Feeding Chloretone on the Blood Plasma Ascorbic Acid of Bulls

Bull No.	Daily Dose	Days Fed	Ave. of: 10 days: Before	Ascorbic Acid per 100 ml. of Plasma After Initial Feeding of Chloretone — Week 1st	2nd	3rd	4th	5th	6th	7th	8th	9th	Peak
	mg.		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1645	5	20	.280	.300	.430	.520	.310	.360					.520
1645*	5	29	.360		.389	.495	.435		.157	.218		.342	.495
1840**	10	3											
	5	13	.390	.298	.298	.480	.552	.491					.630
Patty	5	42	.220	.280	.412	.510	.270	.397	.350		.079	.123	.510

*25 days after last dose in first experiment.
**dose reduced to 5 grams per day on fourth day.

A study was made of the effects of feeding chloretone over a long period of time. Two heifers were fed five grams of chloretone daily for 60 days. The plasma ascorbic acid level was studied and the animals observed for harmful effects of feeding this compound.

At the end of the 60 days of feeding five grams of chloretone daily both animals appeared to be normal and did not show any evidence of harmful effects from feeding this drug. The "peak" in the plasma ascorbic acid level was reached during the third week in both animals. However, heifer C-424 showed a higher average value for the ninth week as shown in table XV. Although the levels decreased after reaching the "peak" they did not return to the pre-feeding levels until chloretone feeding was discontinued. Two weeks after chloretone feeding was discontinued the trend in the plasma ascorbic acid levels was toward the pre-feeding levels.

Table XV. The Effect of Feeding five Grams of Chloretone per Day for 60 Days

Ascorbic Acid per 100 ml. Plasma	A-32	C-424
	mg.	mg.
10 days before feeding chloretone	.289	.306
1st week of feeding chloretone	.699	.695
2nd " " " "	.869	.714
3rd " " " "	1.012	.743
4th " " " "	.845	.675
5th " " " "	.733	.616
6th " " " "	.746	.712
7th " " " "	.663	.710
8th " " " "	.690	.626
9th " " " "	.670	.789
1st week after discontinuing chloretone	.595	.589
2nd " " " "	.532	.576
Peak	1.017	.302

DISCUSSION OF RESULTS

Normal Blood Plasma Ascorbic Acid Values for Dairy Animals

This study of the blood plasma ascorbic acid level of dairy calves showed that there was a wide variation in the values found. Values ranging between .047 and .549 milligrams ascorbic acid per 100 ml. of plasma were observed. One Guernsey and five Holstein heifers over one year of age showed a range in plasma ascorbic acid values between .263 milligrams and .739 milligrams per 100 ml. plasma. The average values for individual heifers ranged between .401 and .640 milligrams per cent. The values observed for these heifers were about 100 per cent greater than the values found for young calves. These values were also much higher than the average values for cows of these breeds reported by Phillips (105).

The average values for individual calves for the ten week period studied were between .166 and .457 milligrams ascorbic acid per 100 ml. of plasma. When the values observed were averaged for each week a range between .247 milligrams per cent for the first week and .409 milligrams per cent for the tenth week was found. Since only three animals were observed during the tenth week and two of these were calves that had been showing high ascorbic acid values this average value should not be interpreted as being an increase in plasma ascorbic acid due to the ages of the calves. The average value of .314 milligrams per cent obtained for the eighth week is more nearly accurate. These data indicate that the plasma ascorbic acid level increases from about .247 milligrams ascorbic acid per 100 ml. of plasma during the first week to .314 milligrams ascorbic acid per 100 ml. plasma in the eighth week.

The number of animals used in this investigation was insufficient for determining a breed difference in the plasma ascorbic acid values.

All the cows used in this study were milking Holstein cows on rations made up of corn silage, alfalfa silage, alfalfa hay and in some cases supplemented with various concentrates. A total of 43 values was used for calculating the average plasma ascorbic acid values. Values between .200 and .668 milligrams per cent were observed. Phillips (105) in a study of 85 cases reported an average of .350 milligrams per cent with a range of .190 to .600 milligrams per cent for Holstein cows. The average of plasma ascorbic acid values for these cows was .468 milligrams per cent and the averages for the individual animals ranged between .385 and .591 milligrams per cent. Wallis (106) found average values between .320 and .446 milligrams per cent plasma ascorbic acid for barn fed cows. One possible explanation for the average values obtained in this study being higher than those reported by other workers may be the fact that two of the cows observed showed values approaching the maximum of the range reported while none of the animals showed values near the lower limit.

The extreme variations found in the plasma ascorbic acid values of individual animals of all ages suggested that there was either a normal daily variation in the plasma ascorbic acid or else the variations in the values obtained were due to errors in the method of analysis.

An experiment was set up to check the method of analysis. Five animals were bled at three hour intervals for a period of 24 hours. A large blood sample was drawn at the first bleeding and the ascorbic acid content determined, in addition ten ml. of filtrate were prepared and held as check. Ten ml. of plasma were also retained and the ascorbic acid value determined at three hour intervals. The small variation in the ascorbic acid values obtained for the original filtrate at three hour intervals indicated that

the method of analysis was fairly accurate and that the variations in the values obtained for blood samples taken at three hour intervals were due to actual variations in the ascorbic acid content of the plasma and not to errors in the method of analysis. It was also shown that plasma filtrates may be held for as long as 24 hours without an appreciable loss in the ascorbic acid content. This study also showed that the ascorbic acid was less stable in plasma than it was in the filtrate prepared from the plasma. Phillips (105) also observed that plasma filtrates could be held for as long as 24 hours without significent losses of ascorbic acid. Plasma held in the ice box for 15 hours showed an appreciable decrease in ascorbic acid content. These findings on the stability of ascorbic acid in plasma held in the ice box are in close agreement with those reported by Greenberg and Rinehart (15).

Since it was definitely shown that wide variations did occur in the plasma ascorbic acid, a study was made to determine how great these variations were and if possible determine the normal range. To determine this range cows were bled at the same time each day and the blood samples handled by exactly the same procedure to eliminate any possibility of variations due to the method of analysis. Five of the cows were bled each day for ten days and three cows were bled each day for eight days. The average value obtained for this group of cows was .402 milligrams per cent ascorbic acid and ranged from .290 to .599 milligrams per cent for individual cows. These values are in close agreement with the range reported by other workers. The average of the minimum values obtained for individual cows was .266 milligrams per cent and the average maximum values obtained for individual cows was .569 milligrams per cent. The widest variation found for an individual

cow was that of cow A-23 with a minimum plasma ascorbic acid value of .156 milligrams per cent and a maximum value of .592 milligrams per cent. The narrowest range observed was for cow 267 which had a minimum value of .325 milligrams per cent and a maximum value of .552 milligrams per cent ascorbic acid. Of the 74 values observed for these eight animals the following distribution of values was found: four values between .100 and .200 milligrams per cent, 14 values between .200 and .300 milligrams per cent, 25 values between .300 and .400 milligrams per cent, 12 values between .400 and .500 milligrams per cent, 16 values between .500 and .600 milligrams per cent, one value between .600 and .700 milligrams per cent and two values between .700 and .800 milligrams per cent.

Since it was definitely shown that there was a marked variation in the level of plasma ascorbic acid from day to day an experiment was set up to determine whether the time of day that the animals were bled had any effect upon the values obtained. In the first experiment blood samples were taken from 14 animals at three hour intervals and analyzed for ascorbic acid. This study showed that the variations which occurred during the day were as great as those which were observed from day to day. There was no consistency in the time of day at which the minimum or maximum value for the day occurred. Maximum values for one or more animals were observed at all but two bleeding intervals. Minimum values were observed for one or more animals at every bleeding interval. During the 24 hour period studied one animal showed a difference of less than .100 milligrams per cent between her minimum value and her maximum value; eight showed differences of .100 to .200 milligrams per cent, three showed differences of .200 to .300 milligrams per cent and two showed differences of .300 to .400 milligrams per cent.

The smallest difference was .099 milligrams per cent and the greatest difference was .368 milligrams per cent. When the bleeding interval was increased to six hours and the variations for a period of 48 hours were studied it was found that the time at which maximum and minimum values occurred varied from day to day for the same individual. One cow showed a minimum value at 7:00 a.m. and a maximum value at 1:00 p.m. the first day, the second day the minimum occurred at 1:00 p.m. and the maximum at 1:00 a.m. The other animal used in this study showed variations of a similar nature. These results indicated that there was no regularity in the time nor direction in which the plasma ascorbic acid level varied. It was concluded from this study that changes in plasma ascorbic acid values during various feeding experiments were not significant unless the level was changed for a period of several days.

The work of Greenberg and Rinehart (15) suggested the possibility that some of the extremely low values obtained in the previous experiments might have been due to destruction of ascorbic acid caused by hemolysis of the blood sample. When the previous experiments were carried out it was observed that some of the samples showed hemolysis but they were not discarded.

An experiment was set up to determine whether the same variations occurred when blood samples did not show hemolysis. It was found that hemolysis of samples could be prevented by improving the bleeding technique, using the anti-coagulant in solution, and avoiding excess shaking when taking the samples. Four animals were bled at three hour intervals and the samples were observed for hemolysis before determining the ascorbic acid content. Since none of the samples showed any signs of hemolysis it was concluded that the low values obtained in this study and in the previous studies actually represented the level of plasma ascorbic acid.

The Effect of Various Feeds on the Blood Plasma Ascorbic Acid Level

Hjarre and Lillengen (100) reported that they produced a vitamin C deficiency in calves while Thurston and associates (98)(99) found that calves developed normally on vitamin C-free rations. The results of this investigation are in agreement with those of the latter investigators. It was found that calves fed milk low in ascorbic acid developed normally and maintained a plasma ascorbic acid level similar to that of calves fed whole milk containing 15 to 20 milligrams of ascorbic acid per liter. The calves used on this experiment were between the ages of one week and six months which indicated that calves of all ages are capable of synthesizing enough ascorbic acid to maintain a normal level in the blood plasma.

In studying the effect of the ration on the plasma ascorbic acid level various concentrates including beet pulp, corn, soybean oil meal, meat scraps, and tankage were compared with alfalfa hay. The effects, if any, that these feeds had on plasma ascorbic acid were so small that they were difficult to recognize when masked by the wide daily variations occurring in all animals. However, the trend in the plasma ascorbic acid level was slightly downward when the animals were changed from a ration containing roughage to one made up of concentrates.

A basal ration made up of corn sugar, starch, rice krispies, cellulose, and salt was also compared with alfalfa hay. The two animals used in this study showed changes in their plasma ascorbic acid level which were great enough to be significant.

The relationship of vitamin A to vitamin C in the dairy animal is not definitely known. Phillips and associates (95) and Sutton (107) reported

that dairy animals on a low level of vitamin A may also be deficient in ascorbic acid. These workers are of the opinion that sufficient vitamin A in the ration is essential to maintaining a normal ascorbic acid level. The results of this investigation show that calves fed a ration low in vitamin A had plasma ascorbic acid values within the normal range. However, it was noted that all values for these calves were between the lower limits of the range and the average value reported. It is interesting to note here that the daily variations were much less for the calves fed rations low in vitamin A than those for calves on normal rations. The chief factor in narrowing the range of the variations was the absence of exceptionally high values that frequently occur in animals on normal rations. In severe vitamin A deficiency the plasma ascorbic acid drops markedly. This drop in plasma ascorbic acid was not noted in these calves until many other abnormalities due to the vitamin A deficiency were observed. The relationship between vitamin A and ascorbic acid does not appear to be a direct one.

The rat work reported by Longenecker and associates (82)(83)(85) and by Ritz and coworkers (84) indicated that ascorbic acid synthesis could be stimulated by feeding certain compounds. These workers demonstrated that many compounds were effective in increasing the ascorbic acid output in urine of rats. Dill seed oil was chosen for the first experiment because this oil contained about 60 per cent d-carvone which had been shown to be very effective in increasing the urinary output of ascorbic acid in rats. The animals refused to eat hay or grain when it had the oil poured on it. Two heifers ate hay that had the oil sprayed on it but it was difficult to give doses of more than five to seven ml. per day by this method. When the dose was increased to ten ml. per day it was diluted with an equal volume

of butter oil and fed in a gelatin capsule. The heifers did not show any significant change in the plasma ascorbic acid when fed dill seed oil at the rate of ten ml. per day for 13 days so the dose was raised to 20 ml. per day and fed undiluted at this level for 16 days. The results obtained from this feeding experiment indicated that the feeding of dill seed oil at the rate of five ml. to 20 ml. per day was not effective in changing the plasma ascorbic acid level.

Longenecker and coworkers (85) found that chloretone was the most effective compound for increasing the ascorbic acid excretion by the rat. Consequently, it was selected for the next feeding experiments. The size of the doses fed was varied from five grams per day to 40 grams per day to determine the minimum dose which would be effective and also the maximum dose which could be fed without producing harmful effects. It was found that this drug had a cumulative effect and the amount that could be fed with no apparent harmful effects varied with the individual animal. A 40 gram dose made one cow stagger two hours after she received the dose and the first milking after receiving this dose her milk production dropped from a normal of about seven pounds to less than one pound. The anesthetic effect continued for about 60 hours. At this time her milk production returned to about 50 per cent of normal, and gradually increased until she reached her previous level about one week after this 40 gram dose of chloretone was fed. Doses of 20 grams per day were fed to two cows. No anesthetic effect was noted in these animals until after they had received the second dose. The cow that was milking did not show any decrease in production until after she had received the fifth 20 gram dose. The chloretone was discontinued at this time and milk production returned to normal two days later. Ten gram doses were fed to three cows. No harmful effects were noted in one

of these cows. One cow went off feed and decreased in milk production after receiving five ten-gram doses but no anesthetic effects were observed. The other cow showed a slight anesthetic effect for about one hour after receiving each ten-gram dose. Her milk production continued at the normal level until she had received five ten-gram doses. This cow received a total of ten ten-gram doses and her milk production decreased to about 60 per cent of normal. She did not return to full feed and normal milk production until about two weeks after chloretone feeding had been discontinued. One bull that received chloretone at the ten gram level per day was wobbly after receiving the second dose. This drug was fed to eight animals at the rate of five grams per day with no noticeable anaesthetic effect. Two animals were fed five grams per day for a period of 60 days without any apparent harmful effects.

The plasma ascorbic acid values obtained for these animals fed chloretone indicated that there was a significant increase in the level of ascorbic acid in the blood when doses as small as five grams per day were fed. When only one five gram dose was fed the increase was rather small and may be questionable; however, when three or more five-gram doses were fed a significant increase in the plasma ascorbic acid level was observed. A total of 16 animals was used in chloretone feeding experiments. Every animal showed an increase in plasma ascorbic acid. However, one cow did not respond to the chloretone feeding until she was fed this drug for a second period. In the first period this cow received ten grams of chloretone per day for seven days. During this period she went off feed, dropped in milk production, and her plasma ascorbic acid level dropped. When chloretone feeding was discontinued she returned to normal and 11 days after the last

ten-gram dose was fed, she was again started on a chloretone feeding experiment at the rate of five grams per day. She was fed 15 five-gram doses with no harmful effects. This time her plasma ascorbic acid increased and she reached a maximum of over one milligram per cent.

The time when the "peak," which was the highest single value observed, occurred varied with the size of the dose and the length of time this dose was fed. When ten grams or more of chloretone were fed the "peak" occurred within 12 days after the initial dose. When five grams per day were fed the "peak" usually occurred during the third week after the initial dose. Two exceptions to this were found when three five-gram doses were fed. These animals showed their maximum values during the first week after the initial feeding of chloretone. The two heifers that were fed five grams per day for a 60 day period reached their "peak" during the third week of feeding. This "peak" value was an increase of about 300 per cent over the pre-feeding level. During the fourth week after the initial chloretone feeding the plasma ascorbic acid value was about 200 per cent of the pre-feeding value and it continued at this level as long as the chloretone was fed.

The results of this investigation indicate that chloretone may be fed to cows at the rate of five grams per day with no harmful effects. When chloretone is fed at this rate for three days or more the level of plasma ascorbic acid is increased about 100 per cent and will remain at this increased level as long as chloretone feeding is continued. In most cases the plasma ascorbic acid returned to its normal level two to three weeks after feeding the last dose.

These data would suggest the possibility of ascorbic acid therapy by feeding methods. This would eliminate hazards and inconveniences accompanying the injection of ascorbic acid.

SUMMARY AND CONCLUSIONS

1. A study was made of the blood plasma ascorbic acid level of normal dairy animals. The animals used in this study included calves, heifers, and mature cows.

2. There was a significant loss of ascorbic acid in blood plasma held in the ice box for 15 hours or more. Metaphosphoric acid filtrates of blood plasma may be held for 24 hours without a significant loss of ascorbic acid.

3. The average plasma ascorbic values increased from .247 milligrams per cent for calves one week of age to .314 milligrams per cent for calves eight weeks of age. The average values for heifers between 12 and 36 months of age were about 100 per cent higher than those observed for young calves. The average value found for lactating Holstein cows was .468 milligrams per cent.

4. Daily variations as great as 300 per cent were observed for the blood plasma ascorbic acid of dairy cows. The variations in values observed at three hour intervals were nearly as great as the daily variations. These variations did not occur at regular intervals.

5. The data indicated that dairy animals maintained a higher blood plasma ascorbic acid level when they were fed a ration containing alfalfa hay than when they were fed a basal ration. The concentrate that was fed did not have any effect on the plasma ascorbic acid level.

6. Feeding dill seed oil at the rate of 20 ml. per day did not have an appreciable effect on the blood plasma ascorbic acid level of two heifers.

7. Chloretone was fed in doses varying in size from five grams to 40 grams. When three or more five-gram doses of chloretone were fed the plasma ascorbic acid level was doubled.

8. Certain cows that were fed doses of ten grams or more of chloretone per day went off feed and decreased in milk production.

9. Two heifers that were fed chloretone at the rate of five grams per day for 60 days maintained a plasma ascorbic acid level about 100 per cent greater than the pre-feeding level. No harmful effects of feeding the drug were observed.

10. An increase of about 100 per cent was observed in the plasma ascorbic acid level of three bulls that were fed chloretone at the rate of five grams per day.

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