SOME STUDIES ON THE EFFECT OF HEAT STRESS ON THE BLOOD ASCORBIC ACID CONTENT OF CHICKENS

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DECLARATION

This report is the sole work of the author, except where assistance has been sought and acknowledged and it has not been presented any where for any other ggualification.

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ABSTRACT

The effect of heat stress on the ascorbic acid level and glucose content of the blood of chickens were studied.

Under the experimental conditions and temperature range of the study, there was no appreciable change in both the ascorbic acid level and glucose content of the blood of cockerels. This observation was discussed on the basis of the ability of the birds to synthesize their own ascorbic acid to replace the excess used, or that the thermal factor under the given experimental conditions, has no effect at all.

A kinetics method which is still in its infancy was also employed to determine the ascorbic acid level of the blood of the chickens. There is need for further investigations on this.

ABBREVIATIONS

ACTH	-	Adrenocorticotropic hormone.
DNPH	-	2.4-Dinitrophenylhydrazine
DCPH	-	2,6-Dichlorophenolindophenol
AA	-	Ascorbic acid
DAA	-	Dehydroascorbic acid
TLC	-	Thin Layer Chromatography
DKA	-	Diketogulonic acid.
EDTA	-	Ethylene diamine tetra acetic acid.

CONTENTS

CHAPTER ONE

1.0 INTRODUCTION

Many diseases are caused by vitamin C deficiency. Perhaps this is one of the reasons why a lot of studies have been made on ascorbic acid. A disease called scurvy has been known as early as the 15th century. Epidermics of the disease have been reported throughout history and especially during the American Civil War and during the first and second World Wars. The epidermics were however, controlled by utilizing ascorbic acid from fresh vegetables and fruits.

With increased scientific knowledge ascorbic acid, commonly referred to as vitamin C, has been used in the treatment and prevention of the disease.

Abundant information on the subject is available in literature.

In the present work, attempt has been made to study the effect of heat stress on the ascorbic acid level of the blood of chicks. Similar studies on the effect of heat at both low and high temperatures on the ascorbic acid content of some organs of animals have been reported (4, 7, 8, 9, 13).

A Kinetic method has also been employed for determining the ascorbic acid concentration in the blood of the chickens

with the aim of developing the method, which is still in its infancy.

1.1 Research Objective.

It has been known as early as 1923 (28) that birds, unlike man and the guinea pig, are able to synthesize sufficient vitamin C for their normal requirement. They do not, therefore, require additonal vitamin C in their diet. However, it is believed that when they are under some strain, their ascorbic acid level drops due to increased utilization of the vitamin.

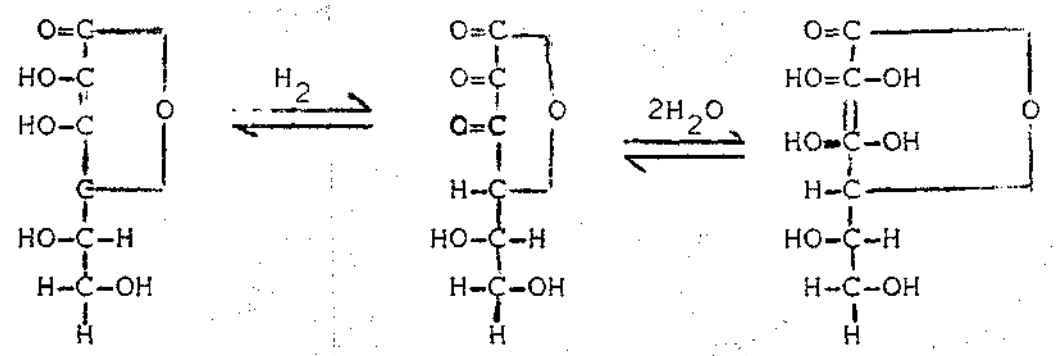
The aim of this research is to apply heat stress to birds at varying temperatures above the environmental temperature and to find out the effect on the ascorbic acid level of the blood of the chicks.

It has also been established (2,3) that ascorbic acid is synthesized from glucose. Thus the glucose content of the blood is also to be determined simultaneously.

1.2 The Chemistry of Ascorbic Acid.

Ascorbic acid is an ordorless, stable white crystalline solid with a melting point at 190^{o}-192^{0}. It is the least stable of the vitamins. This is to say, it is sensitive to alkalis and relatively oxidised in solution to dehydroascorbic acid by mild oxidation by air, H_2O_2, $FeCl_3$, quininone, 2.6 -dichlorophenlindophenol.

The acidity as well as the reducing behaviour of ascorbic acid is attributed to the ionisation of the dienol group. The p.Ka of the first hydrogen is 4.2 and that of the second is 11.6.



Vitamin C (L-ascorbic acid) reduced form

Dehydroascorbic acid (Ketone Structure) Oxidised form

Dehydroascorbic acid (hydrate structure)

Although the vitamin is quite stable in the solid form, it gradually darkens on exposure to light. In the absence of oxygen, the vitamin still remains stable and is not destroyed by heat. However, if air is not excluded, the vitamin is readily oxidised at a rate that increases with rise in temperature.

The vitamin is fairly stable in acid solution. To avoid its oxidation to dehydroascorbic acid, it can be stabilised in solution by EDTA or acetic acid or meta -phosphoric-acetic acid.

Very rapid deterioation of the vitamin takes place in presence of traces of copper, or silver as catalyst but iron and manganese though do not catalyse the autoxidation, accelerate the action of the primary catalysts mentioned above. Destruction of the vitamin by copper can be inhibited by complexing the copper with citric acid. Another distinctive type of catalytic effect is brought by enzymes, heat-labile substances which are common constituents of plant cells. A specific enzyme responsible for the loss of the vitamin is ascorbic acid oxidase a copper-protein complex.

Ascorbic acid is optically active having a specific rotation of $+23^{\circ}-24^{\circ}$ in water and of 48° in methyl alcohol. It absorbs in the uv region at 265, 350 and 400 mU. The maximum absorption band being at 265 mu.

1.3 Sources and Importance of vitamin C.

Analysis of plant and animal tissues reveal that vitamin C occurs naturally in a variety of animals and plants. The ability of plants and some animals to synthesize their own ascorbic acid is discussed later in this report.

The distribution of the vitamin in the animal tissues and plasma is not however uniform. It is reported (1) to be uniquely distributed in certain mammalian tissues.

Tissues like the hypophysis, adrenal thymus, corpus luteum, and retina have several hundred times ascorbic acid concentrations than that of the plasma while other tissues such as the brain, testides, thyroid, small intestinal mucosa, lymph glands, lungs liver, pancreas, salivary glands, spleen etc. contains lesser concentrations. However, ascorbic acid concentrations in the kidney skeletal, smooth and cardiac muscle and erythrocytes are even much lower than those quoted above.

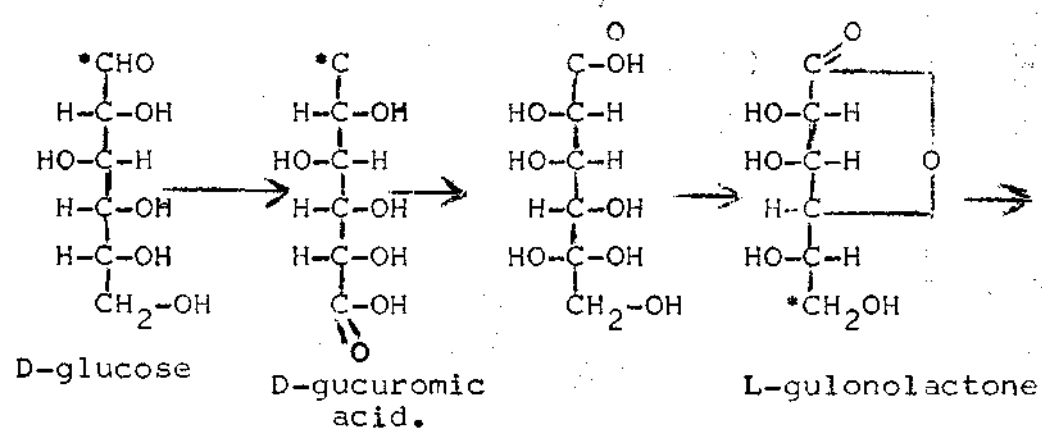
In short ascorbic acid content in animal tissues are greater than that of the plasma. It however occurs in abundance in vegetables such as potatoes, spinach, tomatoes, citrus fruits, pineapples and many others.

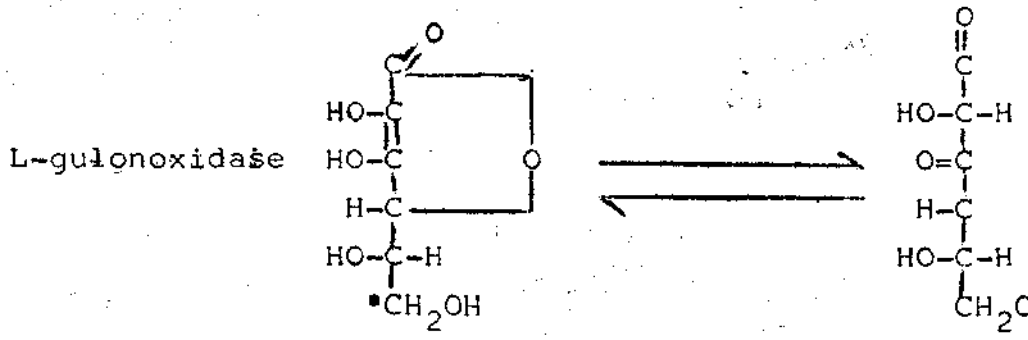
Vitamin C is necessary in human nutrition for proper bone development and calcification. Perhaps the most important use of vitamin C is in the prevention and treatment of scurvy, a disease which arises as a result of vitamin C deficiency. In the industry it has been used in flour treating mixtures, for improving the physical appearance of bread, for preserving natural flavor in milk product and many other uses.

1.4. Biosynthesis of Vitamin C.

It has been established that man, monkeys and guinea pigs are (66) incapable of synthesizing ascorbic acid. The inability of these animals to synthesize their own vitamin C has been attributed to the absence of the enzyme L-gulonoxidase which is responsible for converting the intermediate, L-gulonolactone to L- ascorbic acid.

The path of synthesis, in the rat as proposed by King and co-workers (2) and also confirmed by Mapson (3) and tracer experiments is now believed to take the following scheme.





Where * Denotes the fate of C-1 of D-glucose in this shceme.

However, isotopic studies carried out in the ripening strawberries and germinating cress seedling showed that the vitamin is synthesized from D-glucose by a fairly direct route.

Laboratory methods of preparing ascorbic acid are well treated in the relevant texts.

1.5. Literature Review

Several reports have been made on the effect of heat on the ascorbic acid content of organs of different animals. It appears there is no particular trend, though in most of the cases, the ascorbic acid content decreases with respect to heat stress.

Dobbelstein (4) in investigating the question of increased consumption of vitamin C during fever, reported a drop to half its normal value in the blood of guinea pigs when the animals were overheated in a thermostat. The decrease was explained on the basis of increase in metabolism accompanying the increase in temperature. Thus the tissues utilize more ascorbic acid as the rate of metabolism is increased. In a similar study, at 40°-45°, Martini et al (5), reported a decrease in the amount of ascorbic acid in the liver, adrenal and brain of guinea pigs.

The effect of high environmental temperature on basal metabolism and serum ascorbic acid concentration of women has also been investigated. Under the condition induce by climatic stress with a diminished rate of energy, Thompson et al (6) reported alteration in the ascorbic acid metabolism due to increased requirement or destruction of the vitamin. Differences observed among the subjects were suggested to be due to age or degree of activity and exposure to the direct sun with increased fatigue. In 32-day old albino rats, Irwin et al (7) found that the ascorbic acid content of the adrenal glands subjected to either 3hr cold stress or 1-hr thermopilecold stress were significantly reduced. However, J.W. Woods (8) on exposing rats to freezing temperatures, reported no change in the ascorbic acid concentration in spite of the increase in size of the adrenal glands. But on exposing the rats to cold (-2^{o} to $+4^{o}$) for 1-6 hrs and intense auditory stimulation for 20-120 minutes or surgical operations, he found a depletion of adrenal ascorbic acid in the domesticated rates but not in wild rats.

In another study, the influence of temperature on vitamin levels in bovine blood-a study of B-Vitamin and vitamin C in the blood of calves R Singh (9) and co-workers after rearing Brahman and Santa Gertudis calves at 10^{o} and 26.7^{o} reported that the calves had lower blood vitamin C levels than did the control animals housed in an open shed.

Also Shoji Yamada (10) found the ascorbic acid content in the adrenal cortex of rats to vary according to the thyroid status. This is to say, a higher content was observed in hyperfunction than in hypofunction. But with thermal stimulus at higher temperature, the ascorbic acid content was decreased, the decrease being slightly smaller in the hypofunction than the other cases; while at lower temperature, the decrease was slightly larger in the hyperfunctional case than the others.

In another development, experiments (11) were conducted with Hampshire and China swines in the psychrometric chambers of Herrick Laboratory to study the effect of temperature, as a stressor on the rate of gain and on plasma ascorbic acid levels. Chamber regimens consisted of 29.4^{o} and 85% relative humidity, 18.3^{o} and 85% relative humidity and rotation of animals between these two temperature every 3 days. A quadratic response of the plasma ascorbic acid values to the week on test was found. A significant decrease in weekly plasma ascorbic acid due to the higher temperature without altering the quadratic response was also reported.

Perhaps a more closely related study to this study was the effect of low temperature on the histomorphological, and histochemical changes in chicken adrenal glands reported by Zobundzija and co-workers (12). They investigated the influence of low temperature on the size of cell nuclei and

the concentration of ascorbic acid and other substances in the adrenal glands of 3-4 weeks old cockerels. The birds were divided into the following groups. (a) control (b) control with addition of vitamin C in the food (c) cockerels chilled to -1^{o} for 2hrs during 3 days (d) chilled cockerels with addition of vitamin C in the food. The ascorbic acid concentration decreased remarkably in (c) in both the adrenal and interrenal parts but at the sametime its concentration in (b) and (d) was as in (a).

Changes of ascorbic acid content in the adrenal cortex of rats under the combined action of prolonged physical stress and changes of environmental temperature has also been investigated. In administering repeatedly, different stresses to rats, Ekke et al (13) reported an additional lowering of ascorbic acid content in the adrenal cortex only when ACTH was administered before the second stress. Swimming at 30^{o} for 4.5hr and exposure to 7^{o} for 18hrs were used as stresses. The depression of adenohyphophyseal was suggested as a possible explanation.

Another closely related study to the present work is that reported by A. Ya. Tseitina (14). After exposing rats to high temperature 35^{o}, the ascorbic acid, dehydroascorbic acid and diketagluconic acid were determined in various organs of the rats. In Parallel experiments, the diet was

supplemented with 5mg vitamin P (tea cate chins). In rats exposed to heat, the ascorbic acid in the adrenals and blood serum decreased as compared with rats kept at 20°-22° while in the liver it practically remained unchanged and increased in the urinary. The addition of vitamin P to the diet had somewhat normalizing effect on the ascorbic acid content of the adrenals, and blood serum while enhancing still further the urinary excretion. The dehydroascorbic acid and diketogluconic acid contents in the organs were also reported. It was concluded that vitamin P as well as heat stimulates the synthesis of ascorbic acid.

The effect of artificial light on the vitamin C content in plants has also been studied. G.M. Katcenkov et al (15) found in candescent lamp light to increase the vitamin C content by 48%. Neon lamps (lacking in green-blue-violet rays) either decreased or did not affect the vitamin C content.

In a study of change of vitamin C content in young chickens in experimental malaria, L.M. Bremener and co-workers (16) infected young chickens intramuscularly with sporozoits from Aedes aegypti or with blood from a chicken infected with parasites of plasmodium gallinaceum. He fond that vitamin C in certain infected organs to increase while that in the blood decreased.

In the organs of the chickens at the end of the incubation period, no increase in vitamin C was found.

Similarly no change was also found in the organs of the chickens at the end of the investigation (except in the spleen). At the height of the infection, vitamin C increased in the kidneys, liver, and spleen. The increase was said to have been due to the direct reaction of the organism on the pathological process.

In the same organs of guinea pigs suffering from malaria, vitamin C decreased because the guinea pig is unable to synthesize ascorbic acid. Thus the biosynthesis of vitamin C increases during malaria fever due to its greater need. In a similar study (17) cecal coccidiosis, infections, and experimental hemorrhage, the adrenal ascorbic acid levels in the chicken was found to increase remarkably from 156 to 207mg% in chickens with acute coccidiosis, this was duplicated by experimental hemorrhage.

In studying the histochemical reactions in the suprarenal gland and the ascorbic acid concentration in the blood serum of hens, Bego and co-workers (18) reported an increase in the serum ascorbic acid (1.29mg%) in the passive hens as compared to the controls.

Also in the control group as well as in the active hens (cannibalistic) the intensity of the histochemical reaction as well as the serum concentration of ascorbic acid were also increased (1.13mg%). In another study, Moorhead and co-workers (19) reported no significant alteration in blood ascorbic acid levels of chickens with sulfa-quinoxaline-induced aplastic anemia with either the poisoning or by L-ascorbic acid supplementation in the diet.

The proceeding paragraphs deal with some of the reports made on the relationship between ascorbic acid and other vitamins and substances in organs of animals.

Contrary to reports made on cattle and rats, vitamin A deficiency has been found (20) to have no interference with the ascorbic acid synthesis in the chicken. Thus any depression in the chicken may not be associated with vitamin A deficiency. However a deficiency of pterogulamic acid results in an increase in the concentration of vitamin C. The introduction of pteroglutamic acid or pteroylaminozelaic acid helps to retain the vitamin in the spleen of chicks at a normal level, (21).

Since ascorbic acid is synthesized through the intermediate glucuronic acid, the effect on thiamine deficiency on the intermediate compound has also been studied.

It has been found (22) that a deficiency in thiamine results in a decrease in the conversion of glucuronic acid to vitamin C while the addition of thiamine to diet increased the ascorbic acid synthesis in the rat.

In children the intra-muscular administration of vitamin B_1 has been reported (23) to lower the ascorbic acid content of the blood while it increases the urinary ascorbic acid. The reverse situation was also found to be true. But in guinea pigs, vitamins A, B_1 and D_2 have been found (24) to have no effect on the ascorbic acid reserves of the animals on a scorbutogenic diet. Also in guinea pigs, the vitamin C content of blood has been reported (25) to increase after the injection of amino-pyrine or codeine. The effect being greater with amino-pyrine. The additional ascorbic acid was suggested to have come from either the vitamin reserves or is produced from the liberation of vitamin C which was previously bound to the tissue.

Mossly et al (26) reported that birds suffering from coccidiosis, coryza, fowl pox, epidemic tremor, leucosis, tumors, enteritis etc etc showed no consistent variation in the ascorbic acid content of the blood plasma either as to type of disease, nutrition, age or sex from those of similar birds not so afflicted.

In a similar study (27) of guinea pigs treated with bacterial poisons such as diphtheria and tetanus, do not show any noticeable variation in the ascorbic acid content of their organs although, old tuberculin caused a slight decrease. Experiments (28) on pigeons, chickens, ducks and other animals revealed that these animals do not require vitamin C. The ability of these animals to synthesize their own vitamin C has already been mentioned. Consistent literature values for the ascorbic acid content of chicken blood was not encountered, A.D. Holmes et al (29) however reported that the ascorbic acid content of the blood plasma of rapidly growing chicks, 8 weeks old, averaged 2.03mg%, and that of rigorcus 12 weeks old averaged 2.05mg%. N.S. Schrimshaw et al (30) reported the ascorbic acid level of blood of hens varied at different times from 0.57 to 2.22mg% and that differences between breeds were not consistent. And that the ascorbic acid level of blood did not appear to be related to the levels of vitamin A, carotene or protein. In the present work, the ascorbic acid level of blood of 30 weeks old cockerels was found to be 13.10ug/ml of blood in the morning and 9.98ug/ml of blood in the afternoon. Differences may have been due to biological activity of the birds or climatic factors.

In human blood, mean values of 0.884mg/100ml and 0.507mg/100ml in women and men respectively have been found (31). R.A. Simmonds reported (32) that New Hampshire and Australorp chicks tended to have higher blood ascorbic acid than white Legharus especially in the 10-15 week period. He also found significant variation between individuals among the New Hampshires.

S. Maichida and co-worker (33) found the distribution of ascorbic acid in organs of the hen to be 0.014-0.0295% in brain, liver and spleen, 0.005-0.010% in kidney, heart and bile', 0.0003% in eggyork and that in egg white could not be proved to be present.

Although rats like chickens are able to synthesize vitamin C, J.I. Svirbey (34) reported that adequate amounts of vitamin B factors are essential to obtain normal values for the concentration of vitamin C in the organs of the rat. As the metabolism of the rat is increased, the utilization of ascorbic acid by the tissues is also increased. And that the simultaneous adminstration of sodium fluoride and 2,4- dinitrophenol at a level where either substance alone has no appreciable effect on the rat, decreases the vitamin content of the organs.

CHAPTER TWO

2.0 Methods For the determination of Ascorbic acid and Glucose

2.1 Methods For the Determination of Ascorbic acid.

Methods have long existed for the determination of ascorbic acid. They vary from titrimetric to instrumental methods and have been used for both macro and microanalysis. In general the methods could be classified into two broad categories viZ:-

(i) Bioassays

(ii) Chemical Methods.

In bioassays, the optium amount of a food substance required to prevent scurvy in guinea pigs, represents the relative amount of vitamin C in the sample. Sherman et al (35) reported the first satisfactory bioassays using guinea pigs. Sincer then, a large number of bioassays have been reported. A credit to bioassays is that, they measure the true activity of the vitamin, but are time consuming and lack precision. Chemical methods are therefore more generally used.

Chemical methods for the determination of vitamin C are based on its reducing and oxidising properties. The methods frequently used include the following:

(i) Titrimetric Methods.

These methods are the most commonly used. They are based on the oxidation of ascorbic acid to dehydreascorbic acid. Some of the important titrimetric methods include the use of N-bromosuccinimide (36) iodine (37), 2,6-dichlorophenolindophenol (38, 39), phenathiaz-3-one (40), mercury (II) chloride (41), and others.

The 2,6-dichlorophendindophenol method is regarded as the official method in U.S.A. It involves the titration of the dye with ascorbic acid in which the dye is reduced to a blue colour in the neutral or alkaline solution but pink in acid. The method is used primarily for food and natural products analyses. A major limitation of the method is that dehydroascorbic acid which occurrs in foods, does not react with the dye and is thus not assayed.

For assay of the vitamin in higher concentrations, the oxidimetric reagents, iodine is preferred. The oxidimetric titration of vitamin C is however only specific for pure ascorbic acid solutions otherwise is subject to errors especially when other reducing substances are present. It is not applicable in presence of ferrous, stannous ions etc etc

(ii) Photometric Methods.

Several spectrophotometric methods (42, 43, 44, 45, 46, 47, 48, 49) have been reported.

The 2,4-dinitrophenylhydrazine method (42, 43, 44) is based on the reaction of dehydroascorbic acid with the reagent to produce the hydrazone. When the hydrazone is dissolved in 85% sulphuric acid, a red solution is obtained with a maximum absorption at 515-525 nm. The DNPH (2,4-dinitropheyl-drazine) method could be used for the determination of dehydo-ascorbic acid. In the assay of total ascorbic acid, ascorbic acid and dehydroassorbic, L-ascorbic acid is quantitatively oxidised to dehydroascorbic acid with a suitable oxidsing agent such as norit or bromine.

The method is suitable for the determination of ascorbic acid, dehydroascorbic acid as well as total ascorbic acid in foods and biological materials. A major disadvantage with the method however, is that glucose, fructose and glucuronic acid also react with DNPH to form phenylhydrazones at higher reaction temperatures even at 37^{o}C. If these substances are present in greater concentrations than the ascorbic acid their interferences become significant.

The interferences could be minimised by separating the phenylhydrazones from the ascorbic acid derivative by TLC.

Silica gel plates developed with chloroform/ethyl acetate/ acetic acid in the ratio of 60:35:5. The sharply defined brick-red phenylhydrazones of ascorbic acid is eluted from the silica gel with 85% sulphuric acid and the absorbance is read as before.

In the 2-nitroaniline method (45) the reagent is used to convert the ascorbic acid to oxalic acid 2-nitrophenyldrazide. After the addition of excess sodium hydrazide solution, a reddish violet sodium salt is formed, whose solution absorbs at 540nm. The method is specific and eliminates interferences from other vitamins and compounds that complicate oxidimetric titrations. The method can be used for the determination of ascorbic acid in fruits, fruit juices, cordials, foods fortified with vitamin C and in pharmaceutical preparations.

Since the method is based on the reaction with ascorbic acid, dehydroascorbic acid should first be reduced to ascorbic acid if an assay of total ascorbic acid is desired. In determining total vitamin C, the photometric method with DNPH is simpler.

Recently Stookey (49) reported a very sensitive method using ferrozine. The method was further improved upon by Jalelskis and co-worker (50) for the determination of ascorbic acid in citrus fruits.

The method is based on the reduction of iron (111) by ascorbic acid with the resulting absorbance of iron (11)-ferrozine chelate at 562nm.

(iii) Microfluorometric Method.

The microfluorometric method (51) is based on the reaction between dehydroascorbic acid and o-phenylene diamine to produce a fluorophor of activation maximum at 350nm and a fluorescence maximum at Ca 430nm.

With very low concentration, it can be shown that the fluorescence intensity obeys Beer's law. The formation of boric acid-dehydroascorbic acid complex prior to the addition of the diamine solution is necessary to prevent the formation of a fluorescent derivative of ascorbic acid. Any remaining fluorescence is then due to extraneous material, and serves as a blank.

Ascorbic acid plus dehydroascorbic acid is calculated by comparing fluorescence readings for a sample with readings from a standard curve.

(iv) Chromatographic method (52)

The technique of paper chromatography is used to isolate ascorbic acid from other reducing substances which may likely interfere with the ascorbic acid. The ascorbic acid is extracted from the paper with a suitable element.

In the assay of total ascorbic acid, hydrogensulphide is used to reduce any dehydroascorbic acid that may be present.

A major advantage of determining ascorbic acid after isolating by paper chromatography is that, interferences from undeterminable reducing substances in the titrimetric methods are eliminated.

Quantities as small as 2-3ug ascorbic acid can be detected and the smallest amount that can be determined quantitatively is about 15ug.

TLC using silica gel plates can be used. It has however not found acceptable use because of the difficulty involved in eluting the ascorbic acid quantitatively from the silica gel.

A gas chromatographic method using the trimethylsiyl derivative of ascorbic acid has been reported.(53). It is more convenient, faster and an initial separation of ascorbic acid from other components is not desirable.

(v) Polarographic Method

In this method, the dropping mercury electrode is used.

The electrolyte is a dilute solution of the material under investigation, which must be electro-active, in a suitable medium containing an excess of an indifferent or supporting electrolyte.

When a current is passed into the solution, the current measured is due to the migration of the ions as well as due to diffusion of the ions. In polarography the concentration of the electro-active species is a function of the diffusion current. It is therefore necessary to eliminate the migration current. This isdone by the addition of the supporting electrolyte. The use of the supporting electrolyte is to carry the current in bulk of the solution and to raise the conductivity of the solution. Thus the diffusion current is due to that of the electro-active species. Under this condition, the limiting diffusion current is proportional to the concentration of the electro-active species.

In the polarographic technique a gradual increase of potential is applied. As the applied potential is increased, the current is also increased up to the decomposition potential of the electro-active species. Beyond the decomposition potential, the current no longer increases with applied voltage.

The current at this instance is termed the limiting diffusion current and is proportional to the concentration of the electro-active species in bulk of solution.

This is described more precisely by the Ilkovic equation (54). By keeping all other factors constant except concentration, the concentration of the electro-active species can be determined with reference to a standard curve or by an internal standard method.

Ascorbic acid which displays both oxidisable and reducing properties, can conveniently be determined by polarography. Accurate determinations are possible below 25 ug and up to 250 ug per millitre.

Polarographic methods for vitamin C assay include those by Page et al (55) and that by Krauze and co-worker (56).

(vi) Kinetics Method

Klockow et al (57) recently reported the use of the competitive reaction between ascorbic acid and Mo(VI) catalysed by PO_4 for the determination of the concentration of the catalyst. This method could be further developed for the determination of ascorbic acid.

2.2 Methods of Determining Glucose.

Most common methods deal with the determination of glucose along with other reducing sugars.

Josyln (58) presented a tabular summary of the many sugar methods and modifications at that time. Many of these methods are still in use today but many more have been devised.

It is well known that the estimation of blood-glucose by reducing methods lack specificity and give values which afe too high. Enzymes, however, enable a greater selectivity of the reducing substances to be made. D-glucose is determined quantitatively and selectively (59) in a mixture of sugars with a coupled enzyme system containing glucose oxidase.

In the presence of glucose oxidase, which is highly specific for glucose, glucose is oxidised to gluconic acid and hydrogen peroxide. The hydrogen peroxide can in the presence of peroxidase, oxidise a suitable oxygen acceptor to give chromogenic products, the intensity of whose colour is proportional to the amount of glucose initially present. This method has been applied successfully in blood and urine glucose by Huggett and co-worker (60). The method used in this study is the modified method (61) of Trinder (62) using colour reagent solution of glucose oxidase, peroxidase and 4-amino-phenazone.

CHAPTER THREE

3.1 Experimental Methods and Procedures.

A wooden cage, which housed the chickens, was constructed with an electric heater mounted directly below the floor of the cage. The floor of the cage was covered with wire gauze while some portions of the sides were made of wire-netting. This was necessary in order to allow free circulation of air. Provision was made for feeding and water inside the cage. The cage was then kept in an isolated room.

Before the incubation was bagan, the ascorbic acid content was determined at different times of the day for two weeks. It was found that the ascorbic acid level in the morning is differed from that of the afternoon. The values however remained constant though that of the afternoon was lower. The morning value was chosen as the base-line purely on the basis of convenience.

Before each incubation, the room temperature, cage temperature and the body temperatures of the chickens were measured. Each incubation was began at about 1.00 a.m. to 8.00 a.m.

For each bird, 2mls of blood were withdrawn from the jugular veins and emptied into a sample bottle containing 6mls of 6% trichloroacetic acid as protein extractant.

The syringes were constantly flushed with 10% potassium oxalate as anti-coagulant. After taking the samples, the veins were finally cleaned with absolute ethanol.

The samples were transfered to centrifuge tubes and centrifuged at 2500 rpm for 15-20 minutes. The filtrate was then transfered to 10ml volumetric flasks and used for the analysis.

Samples were taken for not more than three times a week and each temperature repeated twice. The experiment was repeated with another batch of younger chickens.

3.2. Analysis of samples

(A) For Ascorbic acid:

(i) The spectrophotometric Determination using Iron (111) -1, 10-Phenanthroline Reagent (47)

The method is based on the quantitative reduction of ferric to ferrous iron by l-ascorbic acid. The amount of ferrous iron produced which is proportional to the amount of l-ascorbic acid present, is reacted with 1,10-phenanthroline. The absorbance of the coloured solution is measured at 514 mu. The photo-reduction of the ferric complex (iron (111)-1,10-phenanthroline) is avoided by adding EDTA to mask the excessive ferric iron after the colour development.

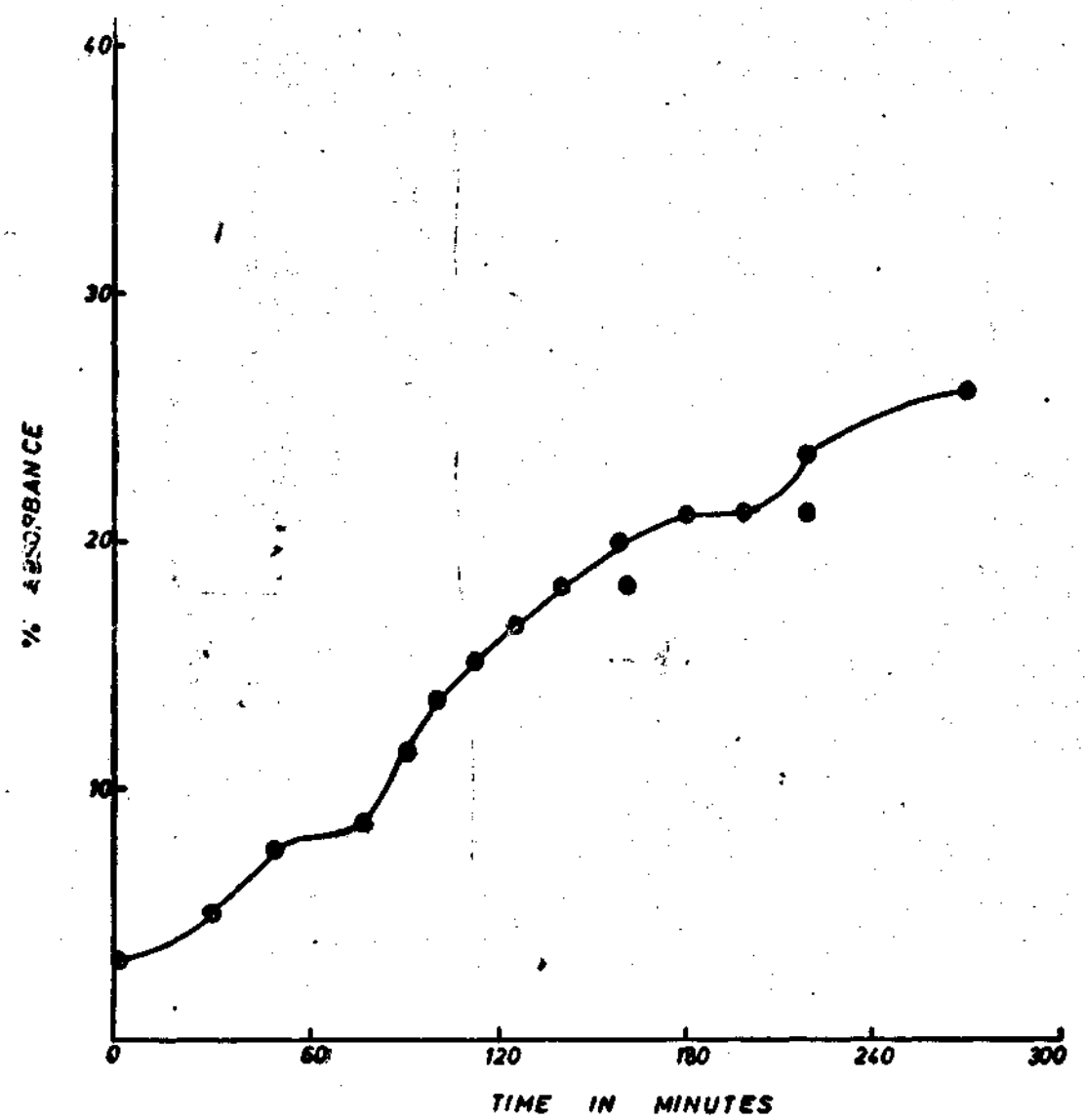


FIG.1: COLOUR DEVELOPMENT OF IRON(II)-1,10-PHENANTHROLIC COMPLEX FOR ASCORBIC ACID IN CHICKEN BLOOD

With pure l-ascorbic acid, the colour development is instantenous. Attempt was made to apply this method to the determination of ascorbic acid in blood. However, the colour development was gradual and too long even when the samples were reduced with E. coli, a bacteria that is specific for reducing dehydro ascorbic acid.

This observation gives the impression either that other reducing species which react much slower than ascorbic acid, are also involed in the reduction process or that the reduction by ascorbic acid is being retarded. This is not unexpected in view of the complexity of blood itself. What these species are, was not investigated. The method was therefore discarded.

To apply this method it would be necessary to isolate the ascorbic acid from other reducing species/ The technique of paper chromatography (52) might be useful if employed along with this method.

(ii) Determination of Plasma Ascorbic acid by 2,6-Dichlorophenolindophenol (63)

Reagents

1. 10% trichloroacetic acid was prepared by dissolving 100g of trichloroacetic acid in 1 litre of distilled water.

2. Stock: 2,6 - dichlorophenolindophenol solution.

40 mg of the dye, weighed accurately, was dissolved in water and diluted to 100ml. One ml of this solution is equivalent to 0.2mg ascorbic acid.

Standardisation.

Since the keeping qualitives of the solution are poor, fresh solutions were prepared at frequent intervals. Each solution was used for not more than one week and each time, standardised with standard ascorbic acid, as follows:

40mg of pure dry ascorbic acid in 100ml acetic acid (100ml glacial acetic acid diluted to 1 litre with water). Dilute 5ml of this solution to 100ml with acetic acid. Titrate 0.5ml of the dye with this solution. 5ml should be required to decolourise it.

2. Working 2,6-dichlorophenolindophenol solution.

Dilute 20ml of the stock solution to 100ml. 1ml of this solution is now equivalent to 40 ug ascorbic acid.

Technique: 0.2ml of the diluted dye was pipetted into small test-tubes and titrated with the filtrate until the reddish colour had disappeared. Analysis were performed in duplicate.

Calculation

Since 0.2ml dye is equivalent to 8 ug ascorbic acid, plasma ascorbic acid in ug/l

$$= \frac{1000}{\text{ml titration}} \times 2 \times \frac{8}{1000}$$

$$= \frac{16}{\text{ml titration}}.$$

(iii) The Determination of Total Ascorbic Acid by the 2.4-Dinitrophenylhydrazine Method (44).

Reagents

Stock ascorbic acid Standard:

100mg of l-ascorbic acid was accurately weighed and placed in a 100ml volumetric flask and diluted to volume with 4 percent trichloroacetic acid solution.

Working ascorbic acid solution: 2ml of stock standard was diluted to 100ml with 4 percent trichloroacetic acid. 1ml = 20ug of l-ascorbic acid.

Trichloroacetic acid: 4 percent and 6 percent solutions were prepared using reagent grade; 2.4-Dinitrophenylhydrazine, 2gm of 2,4-Dinitrophenylhydrazine was diluted in 100ml of 9N sulphuric acid.

It was allowed to stand overnight before filtering through Whatman filter paper.

85 percent sulphuric acid. To 100ml of water 900ml of concentrated sulphuric acid were added.

Thiourea.

10gm of thiourea was dissolved in 50ml absolute ethanol and then diluted to 100ml with distilled water. This reagent keeps for 2 months.

Norit

1 Litre of 10 percent hydrochloric acid was added to 200gm of Norit in a large flask, boiled and filtered with suction. The cake was stirred with 1 litre of water and again filtered. The cake was dried in an oven overnight at 100-120°.

Standardisation.

25ml of working standard l-ascorbic acid was shaken vigorously with teaspoon of Norit for 1 minute, and then filtered through Whatman No. 42 filter paper, 0.0, 0.25, 0.5, 1.0, 1.5, 2.5 and 3.0ml of filtrate were added to test-tubes and each diluted to 4ml with 4 percent trichloroacetic acid which has also been shaken with Norit and filtered. 1 drop of thiourea and 1ml of 2,4-Dinitrophenylhydrazine solution were added to each tube.

The tubes were then placed in a boiling water bath for exactly 10 minutes. At the end of this time, the tubes were placed in crushed ice. 5ml of 85 percent sulphuric acid was added slowly drop by drop and mixed by twirling. The tubes were removed and allowed to stand for 10 minutes. The absorbance of each tube was read against a blank using spectronic 20 at 515 mu wavelength.

A standard Curve was then drawn.

Procedure For Blood Sample.

Preparation of Filtrate.

Whole Blood: To 6 ml of 6 percent trichloroacetic acid, 2ml of blood were added and mixed continously in a sample specimen bottle. It was allowed to stand for 5 minutes and centrifuged for 10-15 minutes at 2500 rpm. The filtrate was poured off into clean dry 10ml volumetric flask and diluted to volume with 6 percent trichloroacetic acid. The filtrate was poured off into clean dry test tubes and ½ teaspoon of Norit was added and shaken vigorously for 1 minute . The solution was filtered through Whatman No. 42 fiter paper. To 4ml of filtrate, 1 drop of 10 percent thiourea and 1ml 2,4 - Dinitrophenylhydrazine solutions were added. The tubes were placed in boiling water bath exactly for 5 minutes.

At the end of this time, the tubes were removed and placed in crushed ice. 5ml of 85 percent sulphuric acid were added slowly drop by drop and mixed by twirling. The absorbance was read as before against a blank prepared in exactly the same manner, except for the omission of the 2,4-Dinitrophenyl-hydrazine until after the addition of 85 percent sulphuric acid.

Calculation.

Volume of filtrate = 10mls.

Volume used for analysis = 4mls

Volume of sample (blood) = 2mls

Amount of ascorbic acid in microgram per millilitre of blood

$$= \frac{\text{Ug ascorbic from standard curve} \times \text{volume of filtrate}}{4 \times \text{Volume of sample}}$$

$$= \frac{Y \times 10}{4 \times 2}$$

$$= \frac{5y}{4} \text{ ug ascorbic acid/ml of blood.}$$

Where y is the amount of ascorbic acid in micrograms from calibration curve.

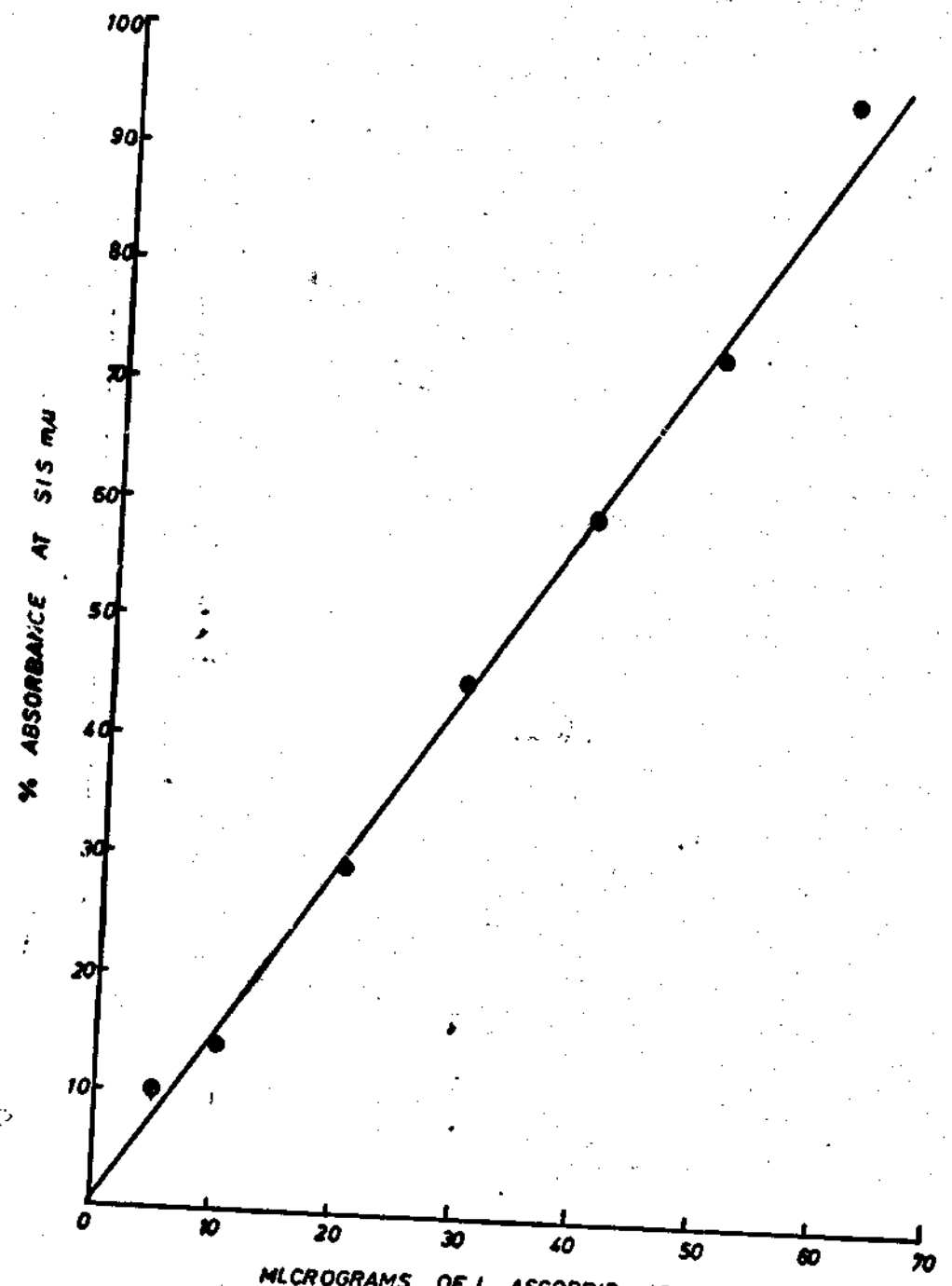


FIG. 2: STANDARD CURVE FOR THE DETERMINATION OF ASCORBIC ACID BY 2,4 DINITROPHENYLHRAZINE REAGENT

(iv) Determination of Ascorbic acid by Kinetic Method.

A method of competitive reaction was applied by Klockow et al (57) to determine the catalyst concentration in a system.

$$\text{Mo(VI)+ascorbic acid} \xrightarrow{K_1 \quad H_2PO_4^-} \text{Mo(V)+dehydroascorbic acid}$$

$$I_2\text{+ascorbic acid} \xrightarrow[\text{(slow)}]{K_{11}} I_2^-\text{+dehydroascorbic acid}$$
(fast)

$$(K_{11} > K_1)$$

Thus in a system where the three components are present together, we have

$$I_2 + \text{Mo (VI)+ascorbic acid} \longrightarrow 2I^- + \text{Mo(V)} + \text{dehydroascorbic acid.}$$

Iodine and Mo(VI) compete for ascorbic acid, the ascorbic acid reaction with the iodine being faster than that with Mo(VI).

In a pre-set rate of addition of titrant (ρ_R = constant) the time t_c (where C implies "competitive") required to remove all of the ascorbic acid ($C_B = 0$) is dependent only on the rate of the slow reaction. It has been shown that

$$tc/t_R = \frac{1}{Q} \text{ In } (Q + 1)$$

where

$Q = K_1 Co_B Co_A \rho_R$ and Co_A is initial concentration of Mo(VI)

Co_B is initial concentration of ascorbic acid.

t_R is the time required to remove all of B (ascorbic aid) in absence of the competitor R, Mo(VI).

N.B. t_c/t_R can only have values $\leq$ 1.

It can be shown further that in a series of experiments where all parameters except C_{cat} are held constant, then Q and consequently t_c are functions only of C_{cat} and thus

$Q = K_1^{11} C_{cat}$. (where C_{cat} is catalyst conc.)

In this way Klockow and co-worker were able to determine the unknown catalyst concentration from a calibration graph of t_c/t_R vs C_{cat}.

In the present work, attempt has been made to keep all parameters constant except the ascorbic acid concentration with the view to determining unknown concentrations of ascorbic acid from a similar calibration graph.

Equipments: Automatic Titrator, Model (Radiometer Compenhagen): pH meter 26, Titrator TTT11, Autobureete ABUII, Titrigraph Type SB2C.

Reagents.

A 0.06M solution of Mo(VI) in dilute sulphuric acid prepared by dissolving 1.451g $Na_2MoO_4 \cdot 2H_2O$ and making up to 100ml with 0.6M sulphuric acid. The solution is allowed to stabilise for one day before use.

A stabilised 100 ppm ascorbic acid solution was made by dissolving 0.1g of analar grade of 1-ascorbic acid and 0.05g of EDTA in 100ml of doubly distilled water.

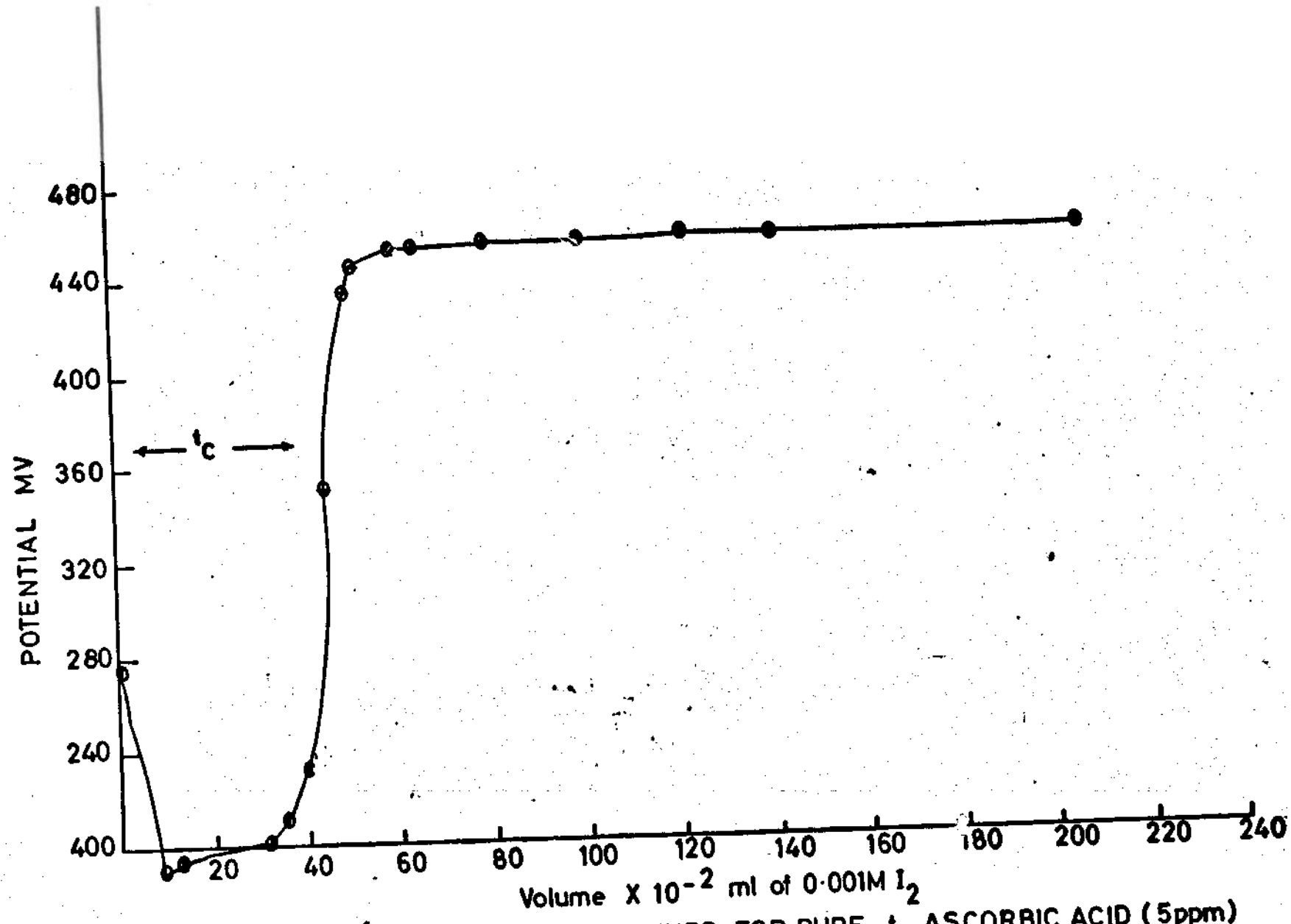


FIG. 4 MANUAL TITRATION CURVE OBTAINED FOR PURE t-ASCORBIC ACID (5ppm)

If stored in darkness, this solution is stable for two weeks.

A 0.001M I_2 solution is prepared daily from a 0.05M stock iodine solution.

100-ppm orthophosphate solution was prepared by dissolving 0.4393g of KH_2PO_4 in 1 litre of doubly distilled water.

Preliminary Work: Determination of Instrumental parameters.

A manual titration with 0.001M I_2 was first performed. The manual titration curve was then plotted. This is necessary in order to determine the direction of the reaction i.e. whether the potential goes upscale or downscale during the course of the reaction.

It was found to be upscale and this was confirmed by an automatic titration curve.

The potential at the end-point was found to be 365 mv. This was obtained with 0.05 proportional band, zero compensation at a chart calibration of 20 mv/cm and rate of addition of titrant on position 3.

The catalysed reaction was obtained by adding 0.1ml of 100 ppm KH_2PO_4 with a syringe. Larger volumes of lower concentrations of PO_4, lowers the starting potential of the solution and thereby delays the addition of the titrant. Thus the Mo(VI) reaction would not be initiated at the same-time with that of the iodine.

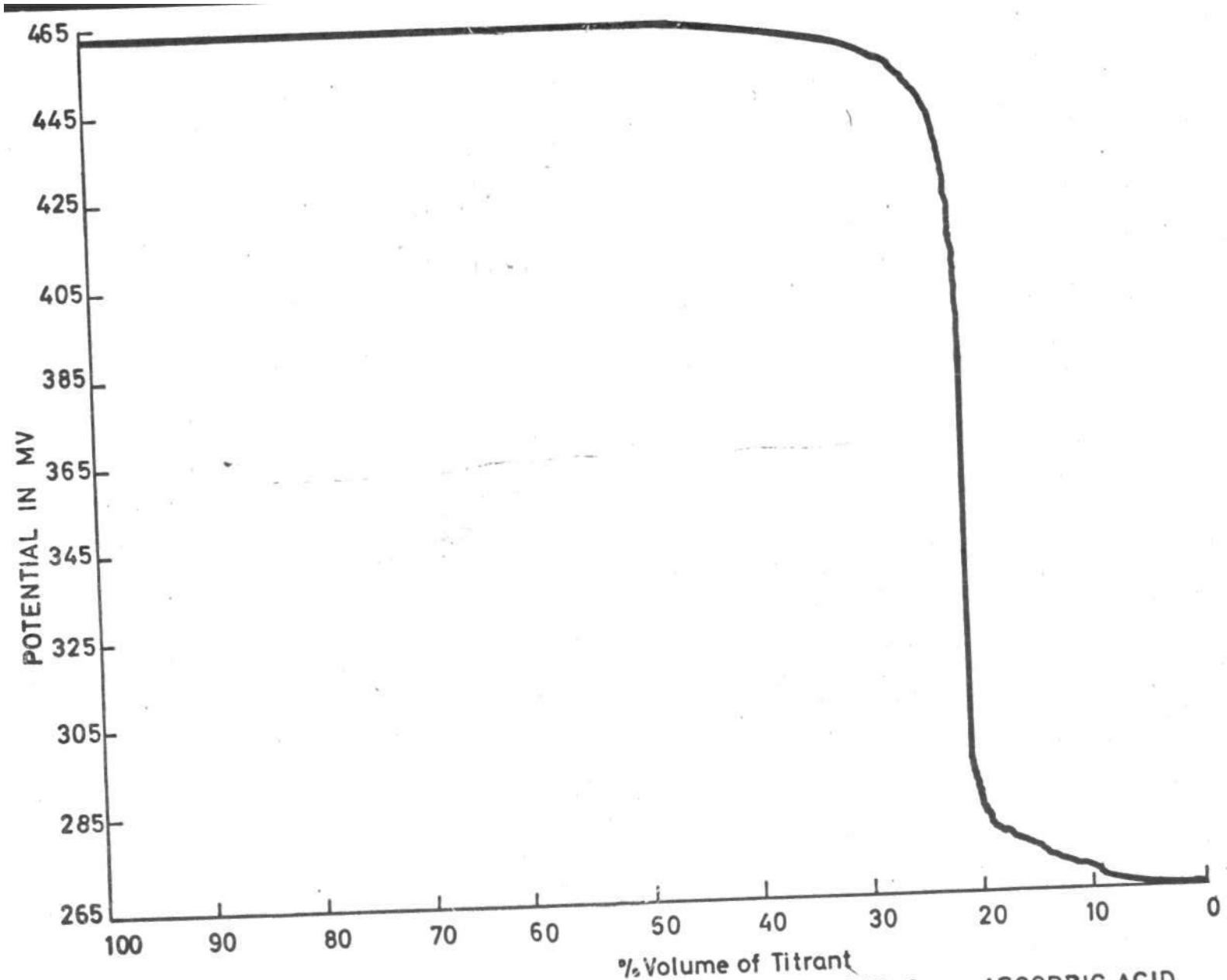


FIG. 5 THE AUTOMATIC TITRATION CURVE OBTAINED FOR 8 ppm ASCORBIC ACID DURING t_c MEASUREMENT

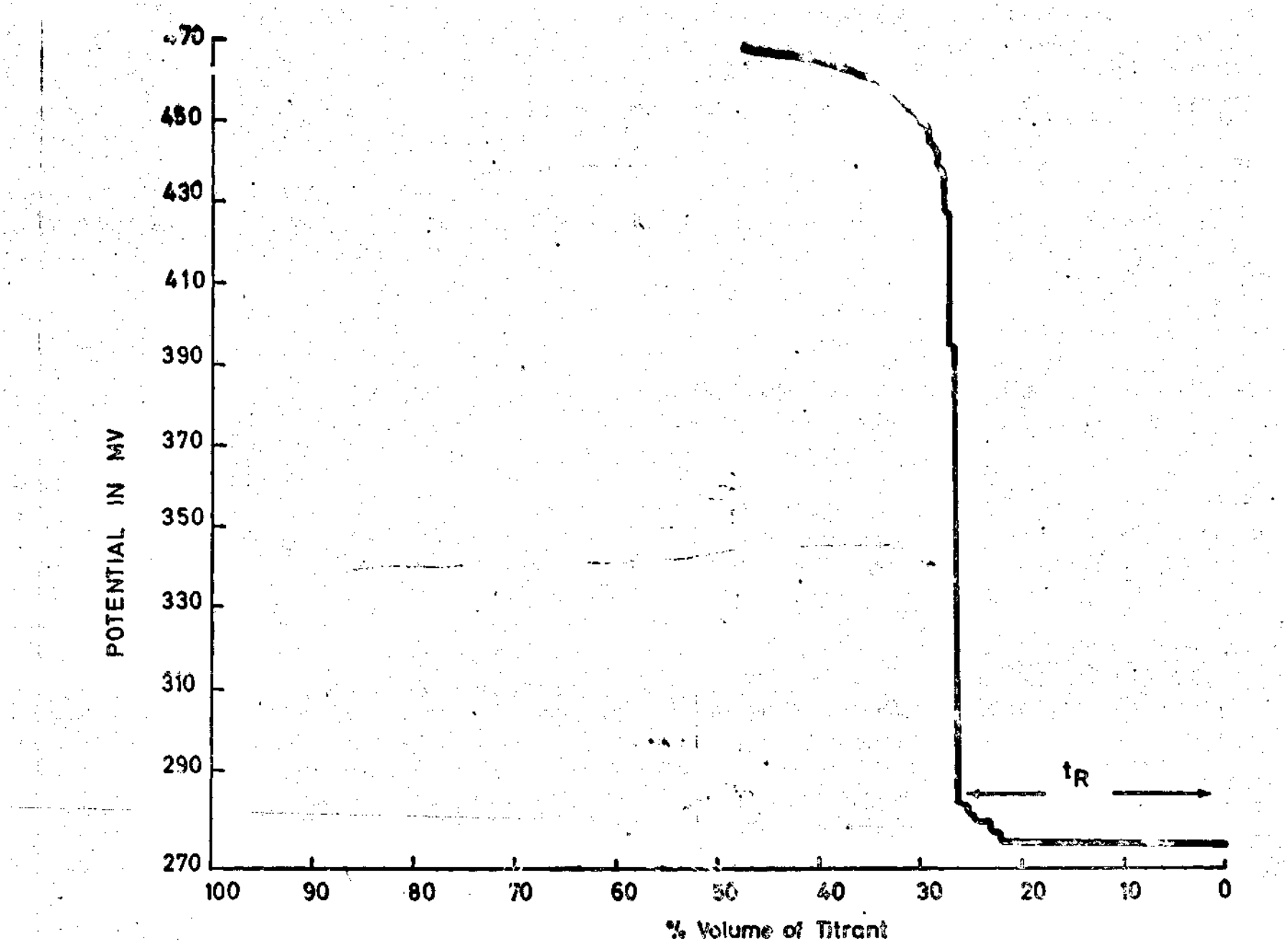


FIG.6 THE AUTOMATIC TITRATION CURVE OBTAINED FOR 8ppm ASCORBIC ACID DURING t_R MEASUREMENT

Treatment of Blood Sample.

A direct manual titration of the blood extract, shows that the reaction was going in the opposite direction with that of the pure standard l-ascorbic.a Figs 4 and 7 This seems to suggest that some other reaction(s) other than the ascorbic acid reaction is/are taking place. Since ascorbic acid in blood plasma occurs mainly in the oxidised form, it was therefore necessary to reduce the acid.

Reduction of Ascorbic acid in Blood Extract

Attempt was made to use two reagents

(i) H_2S

(ii) E. Coli (64) a bacteria specific for the reduction of ascorbic acid.

The manual titration curves after reduction with these reagents are shown in Figs. 8 and 9 The blood extracts were reduced with H_2S by bubbling the gas through the extracts for 15 minutes and allowing the samples to stand overnight.

Although the titration curves, after reduction, follow the same pattern with that of pure l-ascorbic acid, the potenrial at the end-point obtained with the bacteria, is much higher but that with H_2S corresponds.

The t_C measurements for the samples after reduction by bacteria were greater than those of t_R.

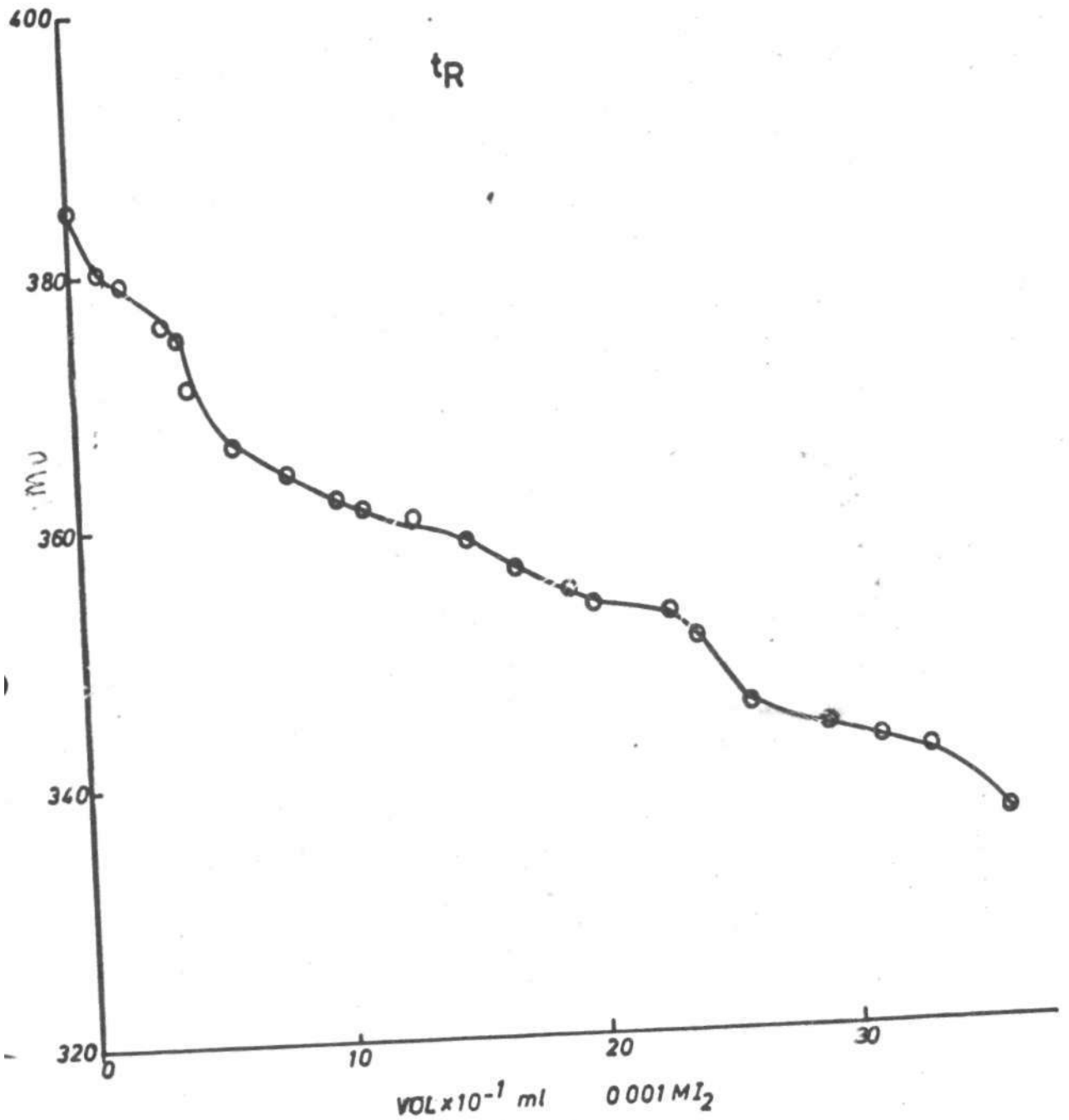


FIG.7: MANUAL TITRATION CURVE FOR ASCORBIC ACID IN BLOOD SAMPLE WITHOUT REDUCTION

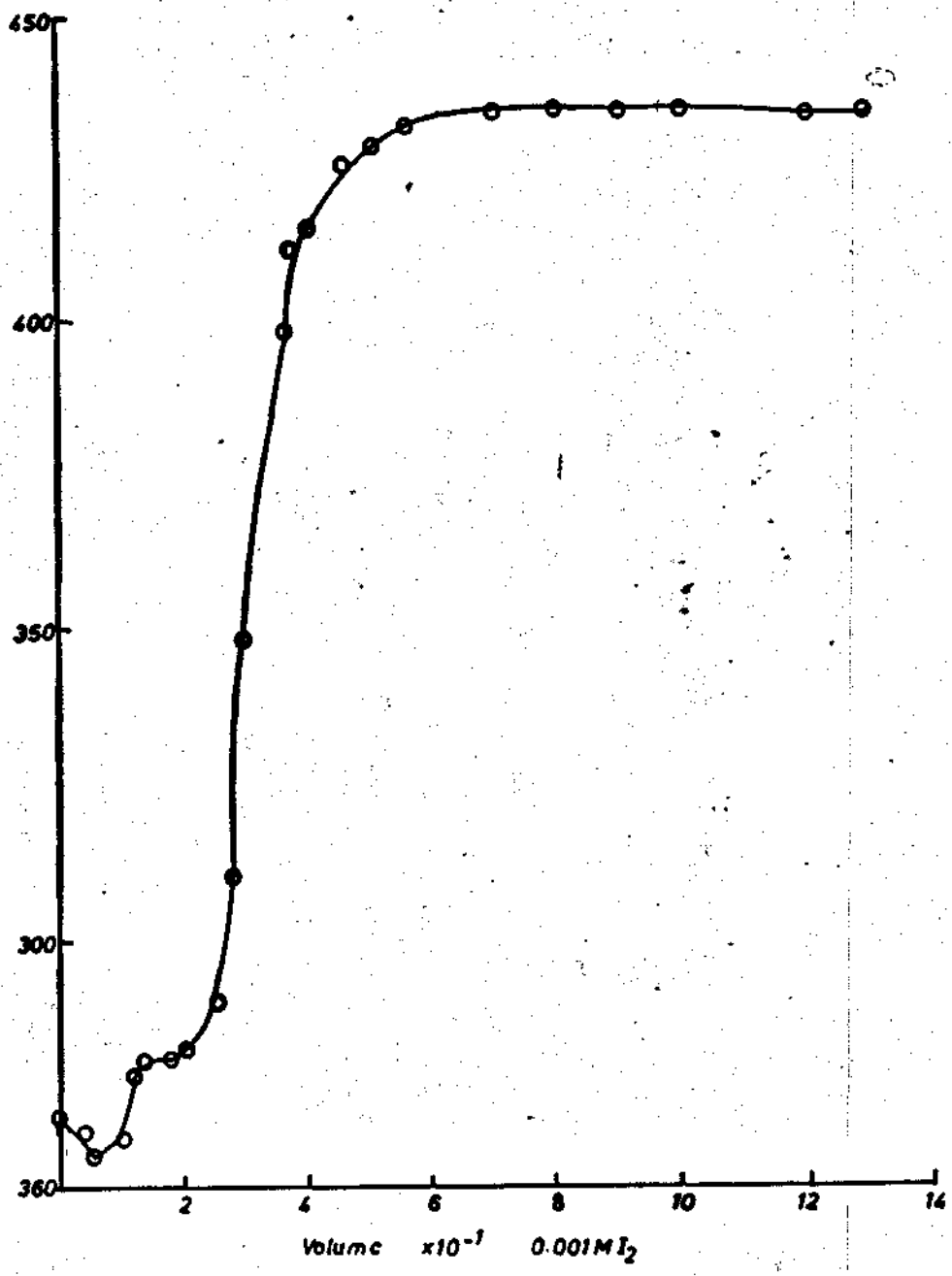
In a situation where there is competition (t_c) we expect t_R to be greater than t_c. The reverse observation made may be explained since at pH 0.7, the Mo(VI) reaction, during t_R measurements, is negligible. But the samples after treatment with bacteria were at pH 5.9 and consequently the Mo(VI) reaction is not negligible which would lead to much shorter t_R measurements.

Standard solutions of l-ascorbic acid were treated in exactly the same manner as the blood samples. The pH was then adjusted to 0.7 with 0.05M H_2SO_4. The end-point potential for each solution was different and this lead to excess titrant in a pre-set automatic titration.

H_sS was chosen as the reducing agent.

Experimental Procedure

A series of standards of 0-20 ppm ascorbic acid were prepared from the stock solution. In a 20ml polyethylene beaker 1ml of Mo(VI) solution is added to 10ml of a standard solution and 20ml of water. A platium and calonel electrodes are inserted into the vessel. The capillary tip of the burette is immersed in such a way that with rapid magnetic stirring the stream of iodine delivered does not directly reach the electrodes. With the end-point set at 365 mv, the catalysed, reaction is initiated by adding 0.1ml of PO_4



FIG·8: MANUAL TITRATION CURVE FOR ASCORBIC ACID IN BLOOD AFTER REDUCTION WITH H_2S

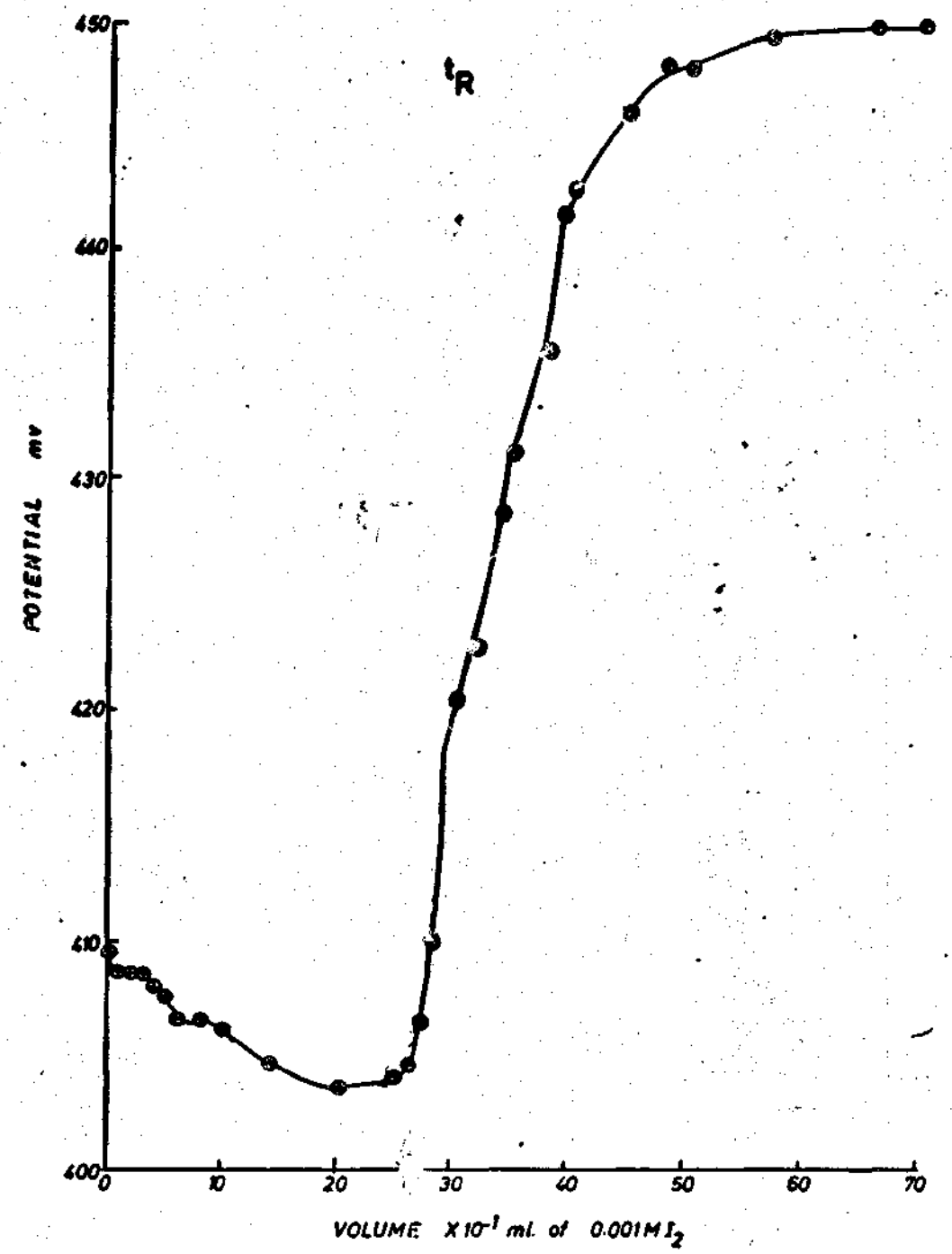


FIG. 9: MANUAL TITRATION CURVE FOR BLOOD SAMPLE REDUCED WITH BACTERIA

and at the sametime the motor-driven burette which delivers the 0.001M I_2 is started. When the end-point is reached, the addition of titrant stops automatically and t_c is directly read from the recorder graph. For each t_c measurement t_R has to be determined. This was easily done by simply using distilled water instead of the phosphate solution. In this case ascorbic acid is used up almost exclusively by iodine because the catalysed reaction between ascorbic acid and Mo(VI) can be neglected at the chosen pH value (pH = 0.7).

The measured times are converted into the dimensionless form t_c/t_R. Unknown ascorbic acid concentrations can be derived from the measured t_c values by means of a t_c/t_R vs ascorbic acid concentration calibration plot.

B. [illegible] For [illegible] Serum Glucose (62, 61).

Reagents

Phenol Reagent

21.25 mmol/L phenol in 0.154M sodium chloride solution was prepared as follows:

Dissolve 1.9998g of colourless phenol crystals in 1 litre of 0.9% sodium chloride (8.9997g NaCl in 1 litre).

Enzyme or Colour Reagent

15ml Fercozyme (glucose oxidase ready solution prepared by manufacturer)

15mg peroxidase.

1.48mmol 4-aminophenazone in 0.1M sodium acetate/acetic acid buffer at pH 6.0 (i.e. dissolve 300mg of 4-aminophenazone in 1 litre of sodium acetate/acetic buffer prepared as follows:

dissolve 12.88g of sodium acetate (anhydrous) in 200ml of distilled water add 0.32ml (320ul) of glacial acetic acid. Make up to volume with distilled water to 1 litre.

Solution keeps for 8 weeks at $4^{o}C$ from date prepared.

Stock Glucose Solution

100m mol/L of glucose: Dissolve 18.02g of glucose in 1 litre of distilled water saturated with benzoic acid i.e. 2.93g of benzoic acid dissolved in 1 litre.

Standard Glucose Working Solutions.

Prepare a series of standard glucose solutions from the stock glucose solution to vary from 0-10m mol/litre i.e. 1ml of stock diluted to 100ml is 1mmol.

0.05ml of the standards will contain glucose ranging from 0-90Ug.

Technique

Add into appropiately labelled tubes:-

	Sample ml	Standard ml	Blank ml
Phenol Reagent	2.5	2.5	2.5
Plasma	0.05	-	-
Standards (0-100mmol)	-	0.05	-
Distilled water	-	-	0.05
Colour Reagent	2.5	2.5	2.5

Mix, incubate at 37^{o} fof 20 minutes, cool, read absorbance at 510mu zeriong with blank.

Calculation.

Glucose in mmoles/Litre is read directly from the standard Curve and converted to mg per ml by multiplying values in mmoles/Litre by 0.9.

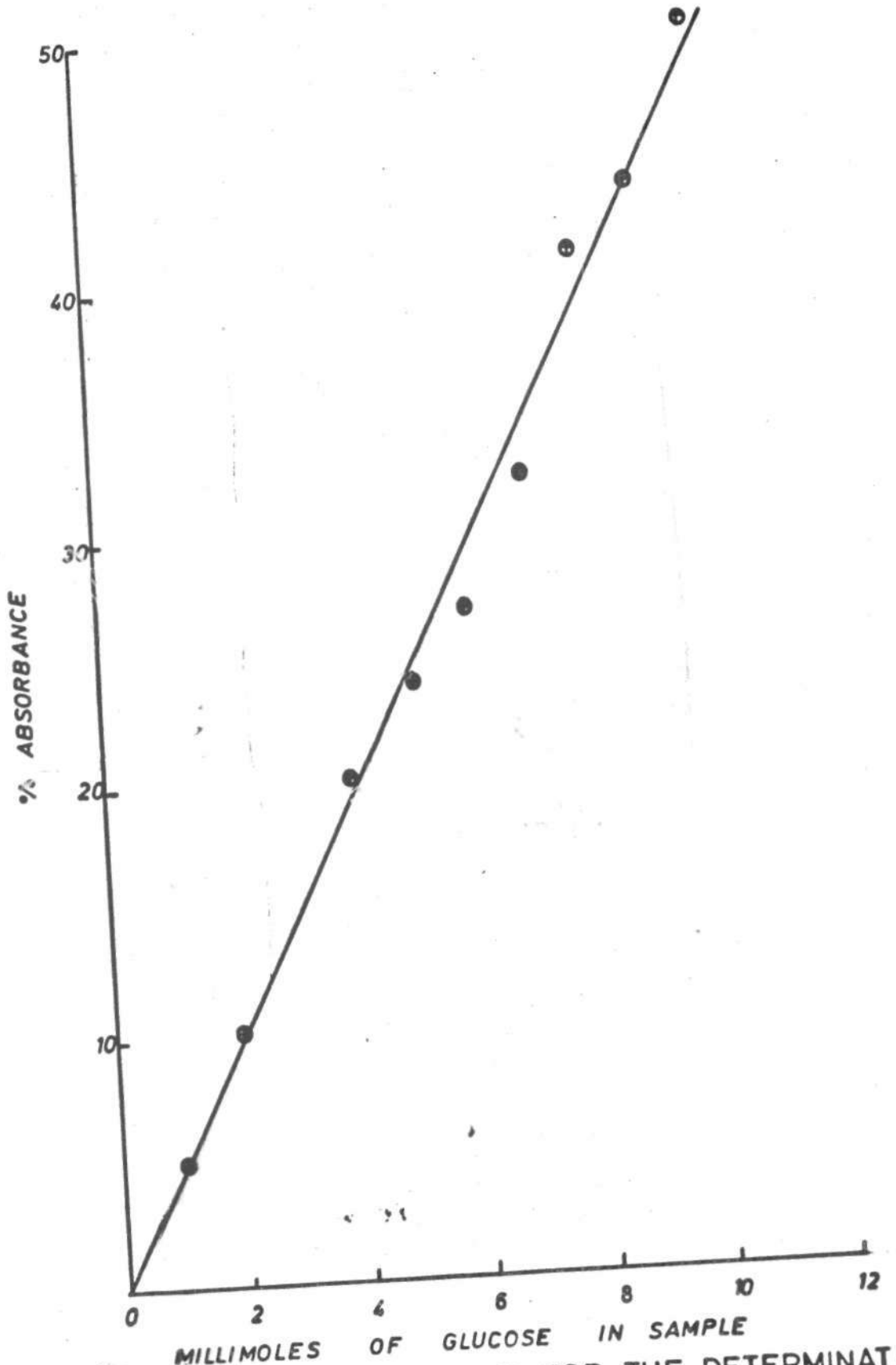


FIG. 3: STANDARD CURVE FOR THE DETERMINATION OF GLUCOSE BY GLUCOSE OXIDASE

CHAPTER FOUR

RESULTS AND DISCUSSION.

4.10 Results

TABLE 4.00: Ascorbic acid levels and Glucose content of the 30 weeks chickens at Different times of day.

IDENTITY OF BIRD	MORNING AVERAGE TEMP. 19°				AFTERNOON AVERAGE TEMP. 28.6°			
	Ascorbic acid µg/ml of blood		Glucose mg/ml of blood		Ascorbic acid µg/ml of blood		Glucose mg/ml of blood	
1	13.10	13.10	1.440	1.080	12.40	10.60	0.900	1.620
2	13.10	13.10	1.350	0.900	10.00	10.00	0.990	1.350
3	13.10	13.10	1.440	0.900	10.00	10.00	1.080	1.260
4	12.50	12.50	1.260	0.900	8.70	10.00	0.720	1.440
5	13.10	13.10	1.170	0.900	8.10	10.00	0.990	1.350
Mean	13.10			1.134	9.98		1.170	

TABLE 4.10: Ascorbic acid levels and Glucose content of 30 weeks old chickens at different temperatures.

IDEN-	26°		28°		30°		37°		40°	
TITY OF BIRD	Ascor-bic acid ug/ml of blood	Glu-cose mg/ml of blood	Ascor-bic acid ug/ml of blood	Glu-cose mg/ml of blood	Ascor-bic acid ug/ml of blood	Glu-cose mg/ml of blood	Ascor-bic acid ug/ml of blood	Glu-cose mg/ml of blood	Ascor-bic ac ug/ml of blood	Glu-cose mg/ml of blood
1	8.70	1.260	8.10	1.170	12.00	1.314	12.50	1.350	10.00	1.350
2	8.70	1.080	7.50	1.260	10.60	1.260	12.00	1.080	10.60	1.440
3	10.00	1.440	8.70	1.350	12.50	1.404	10.60	1.080	8.70	1.800
4	10.00	1.260	8.10	1.260	12.00	1.440	12.00	1.080	10.00	1.620
5	8.70	1.080	8.70	1.080	13.10	1.440	12.50	1.080	10.00	1.440
Mean	9.22	1.224	8.22	1.224	12.45	1.371	12.00	1.134	9.86	1.530

Ascorbic acid determined by 2.4-Dinitrophenylhydrazine Reagent.

TABLE 4.20: Plasma Ascorbic acid levels of 9 weeks old chickens determined by 2,6-dichlorophenolindophenol titration.

IDENTITY OF BIRD	24.5°	23.8°
	Ascorbic acid µg/ml	Ascorbic acid µg/ml
1	7.40	8.00
2	8.00	8.00
3	7.80	7.47
4	6.80	5.86
5	14.30	6.53
Mean	8.86	7.17

TABLE 4.30: Plasma Ascorbic acid levels of 11-13 weeks old chickens determined by 2,6-dichlorophenolindophenol titration.

IDENTITY OF BIRD	30°	41°
	Ascorbic acid µg/ml	Ascorbic acid µg/ml
1	11.18	9.24
2	9.81	7.80
3	8.30	6.67
4	8.00	8.51
5	13.33	9.81
6	10.10	9.70
7	10.66	9.81
8	7.88	8.00
9	11.42	8.74
10	8.18	8.00
Mean	9.87	8.66

TABLE 4.40: Results For t_c and t_R measurements For standard Ascorbic acid solutions obtained with 0.001M I_2 and End-point set at 365mv.

Conc in PPM	t_c in Sec.				t_R in sec.				t_c/t_R
				mean				mean	
1	3	3	6	4	5.5	5.0	9.5	6.67	0.600
2	4	6	2	4	9.0	9.0	8.0	8.66	0.460
3	5.5	4.5	4.5	4.8	12.5	13.0	12.0	12.50	0.380
4	5.5	5.0	3.0	4.5	14.5	15.0	18.0	15.80	0.220
5	3.0	3.0	3.0	3.0	20.0	20.0	20.2	20.10	0.105
6	3.5	4.0	3.0	3.5	22.0	18.0	16.5	18.80	0.186
8	3.5	4.0	3.7	3.7	23.0	21.5	22.2	22.20	0.160
10	4.0	3.0	3.5	3.5	28.0	27.0	27.5	27.50	0.127
20	4.0	3.5	3.5	3.8	51.0	51.0	65.0	56.00	0.068

TABLE 4.50: Results For t_c and t_R measurements For Blood samples of Chickens incubated at 41°C(by kinetics method.)

Identity of Bird	t_c sec		t_c mean	t_R (secs.)		mean	t_c/t_R	Conc. in PPM	Conc. in PPM sample.
1	6.5	6.5	6.5	11.0	11.5	11.3	0.575	1.5	15
2	4.0	4.0	4.0	8.0	7.5	7.8	0.513	2.0	20
3	4.0	4.5	4.3	7.0	7.5	7.3	0.590	1.3	13
4	6.0	6.0	6.0	13.5	14.0	13.8	0.440	2.2	22
5	5.5	5.5	5.5	15.5	15.5	15.5	0.355	3.2	32
6	6.0	6.5	6.5	14.5	14.5	14.5	0.400	2.6	26
7	6.5	6.5	6.5	13.0	13.5	13.3	0.500	2.0	20
8	6.0	6.0	6.0	14.5	14.5	14.5	0.400	2.6	26
9	6.5	6.0	6.3	17.0	16.5	16.8	0.393	2.7	27
10	6.5	6.0	6.3	14.0	14.5	14.3	0.400	2.6	26

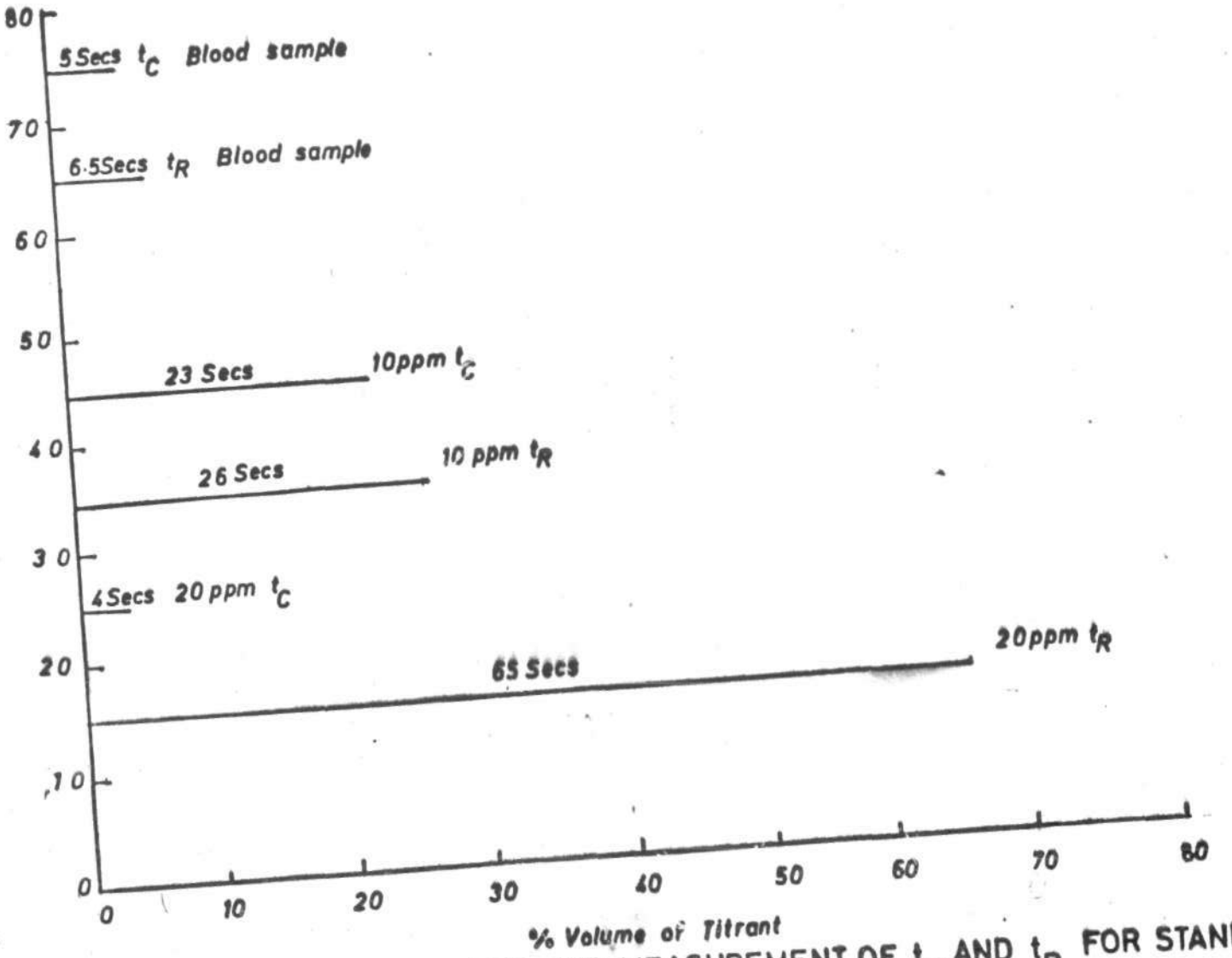


FIG.10 SOME RESULTS FOR THE MEASUREMENT OF t_C AND t_R FOR STANDAR
ASCORBIC ACID BLOOD SAMPLE

TABLE 4.60: Ascorbic acid level of 12-14 weeks old chickens determined by different methods in the same sample.

IDENTITY OF BIRD	TEMPERATURES 24° Ascorbic acid Determined by DNPH µg/ml of blood	24° Ascorbic acid determined by DCPH µg/ml	24° Glucose mg/ml of blood	24° Ascorbic acid by DNPH µg/ml of blood	41° Ascorbic acid by DCPH	41° Ascorbic acid obtained by Kinetics method ug/ml of blood	41° Glucose mg/ml of blood	41° Ascorbic Acid obtained directly from t_R measurements µg/ml
1	8.10	9.70	0.540	8.10	8.64	15.0	1.260	26
2	10.00	10.12	0.540	10.60	9.10	20.0	1.170	20
3	10.60	8.20	0.540	12.50	8.00	13.0	1.080	17
4	10.60	7.44	0.720	10.60	9.70	22.0	1.170	34
5	10.60	8.64	0.540	10.00	10.66	32.0	0.990	38
6	10.60	7.11	0.720	12.50	9.00	26.0	1.260	36
7	8.70	6.60	0.720	12.50	9.10	20.0	1.170	32
8	12.00	8.20	0.720	8.10	8.00	26.0	1.260	36
9	10.00	7.27	0.720	6.25	8.20	27.0	1.260	40
10	8.10	8.42	0.630	6.25	8.00	26.0	0.990	33
mean	9.93	8.17	0.639	9.740	8.840	24.70	1.161	31.2

4.20 DISCUSION

The ascorbic acid level per ml of blood of 30 weeks old cockerels was found to average 13.10µg in the morning as compared to 9.98µg in the afternoon for the same birds. The morning value for 12-14 weeks averaged 8.17µg/ml of blood. From the several determinations made, both the morning and afternoon values remained fairly constant. Since the morning values were chosen as the base-line, determinations in the afternoon for the 12-14 weeks old, was no longer necessary. The lower morning value for the 12-14 weeks old as compared to that of the 30 weeks old, may be partially attributed to difference in ages of the chickens. The glucose content also remained fairly constant within these periods as can be seen in Table 4.0.

Schrimshaw et al (30) reported that the ascorbic acid level of the blood of hens at different times vary from 0.56 to 2.22mg% i.e. 5.7µg/ml to 2.22µg/ml. In another study (29) the blood plasma ascorbic acid content of rapidly growing chicks of 8 weeks old, averaged 2.039mg% and that of vigorous 12 weeks old averaged 2.054mg%. Values of 1.29mg% and 1.13mg% for passive and active hens respectively was also reported (18).

In a biological system where so many factors are involved, the lack of consistent literature values is not unexpected.

However, the results obtained from this study fall within the range of other studies.

With respect to Tables 4.00, 4.10 and 4.60, the ascorbic acid levels at various temperatures were determined by the 2,4-dinitrophenylhydrazine reagent. Tables 4.20 and 4.30 shows the ascorbic acid levels determined by 2,6-dichlorophenolindophenol titration.

When the ascorbic acid levels were determined by different methods in the same sample (Table 4.60), the values obtained by the DCPH (2,6-dichlorophenolindophenol) titration, were generally lower. This is because with DCPH reagent, only the reduced form of ascorbic acid is determined whereas the DNPH reagent, total ascorbic acid (l-ascorbic acid + dehydroascorbic acid) was analysed.

In the kinetics method, a manual titration curve for ascorbic acid in blood sample without reducing the dehydroascorbic acid shows as in Fig. 7, that the reaction was downscale i.e. in the opposite direction to that of pure l-ascorbic acid. This means that an entirely different reaction is now taking place. This is not strange in view of the complexity of blood system. This observation also shows that l-ascorbic (reduced form) is present in very small quantity, the rest being present as dehydroascorbic acid (oxidised form).

Spectrophotometric determination with DNPH reagent also confirms this.

When H_2S or bacteria were used as reducing agents for dehydroascorbic acid, the titration curves obtained, (as shown in Fig. 8 and 9) show that they were of the same pattern with that of pure l-ascorbic acid although the end-point potential of the sample reduced by bacteria did not exactly correspond. Although the bacteria is claimed to be specific for reducing ascorbic acid, the higher potential value of 430mv obtained at the end-point may arise from the medium of preparing the bacteria. The potential at the end-point at the given experimental conditions for pure l-ascorbic acid and blood sample reduced by bacteria was found to be 365mv. In both the pure l-ascorbic acid and blood samples, the reaction initially seems to be downscale. It is difficult to associate any particular reaction with this and no attempt was made to investigate it. However the oxidation reactions of ascorbic acid in solution arising from the equilibrium.

$$\underset{\text{(reduced)}}{\text{AA}} \underset{}{\overset{H_2}{\rightleftharpoons}} \underset{\text{Ketone structure (Oxidised)}}{\text{DAA.}} \overset{2H_2O}{\rightleftharpoons} \underset{\text{hydrate structure}}{\text{DAA}}$$

may be a possible cause.

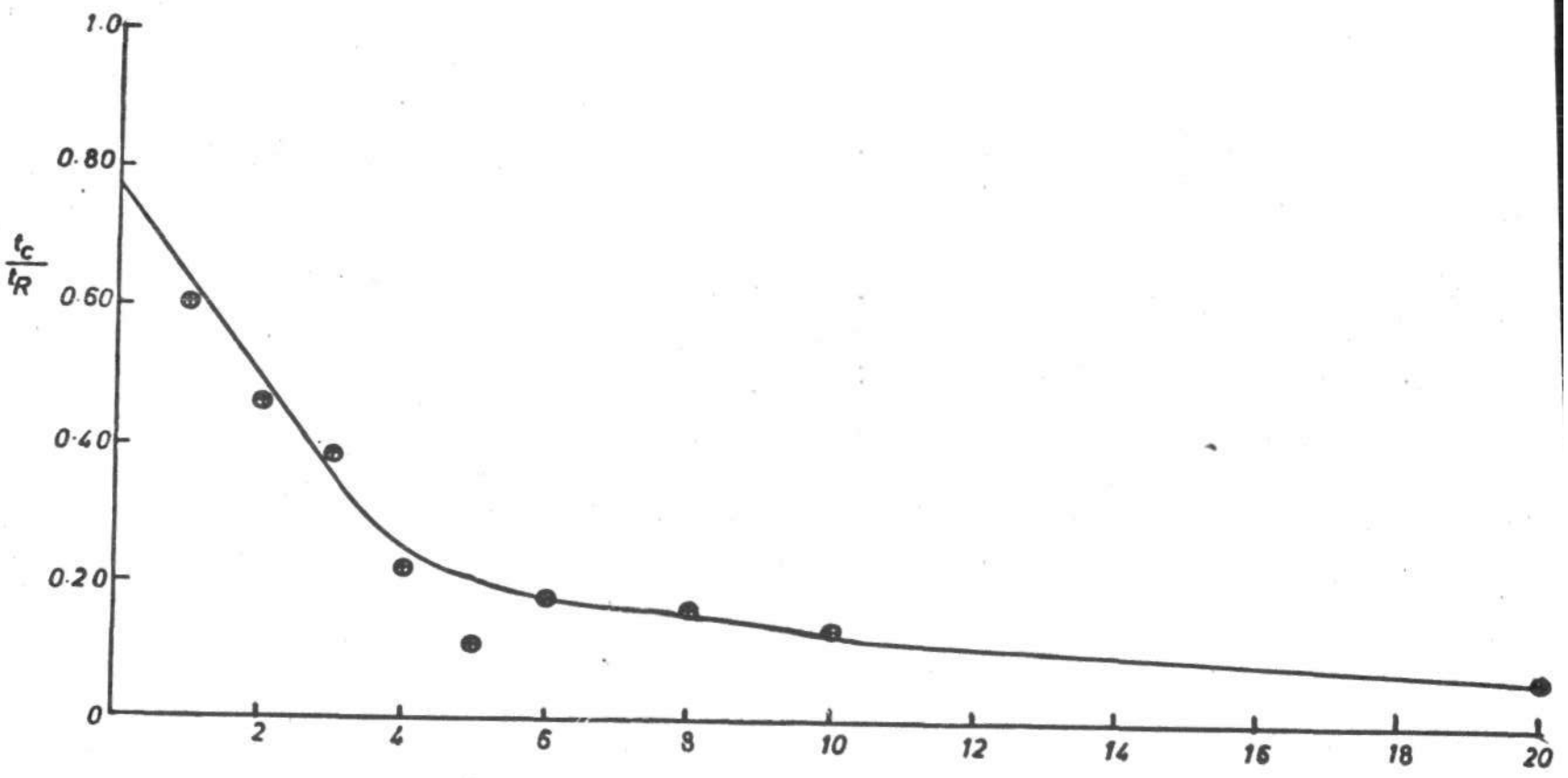


FIG.11: A CALIBRATION CURVE FOR THE DETERMINATION OF ASCORBIC ACID BY COMPETITIVE REACTION

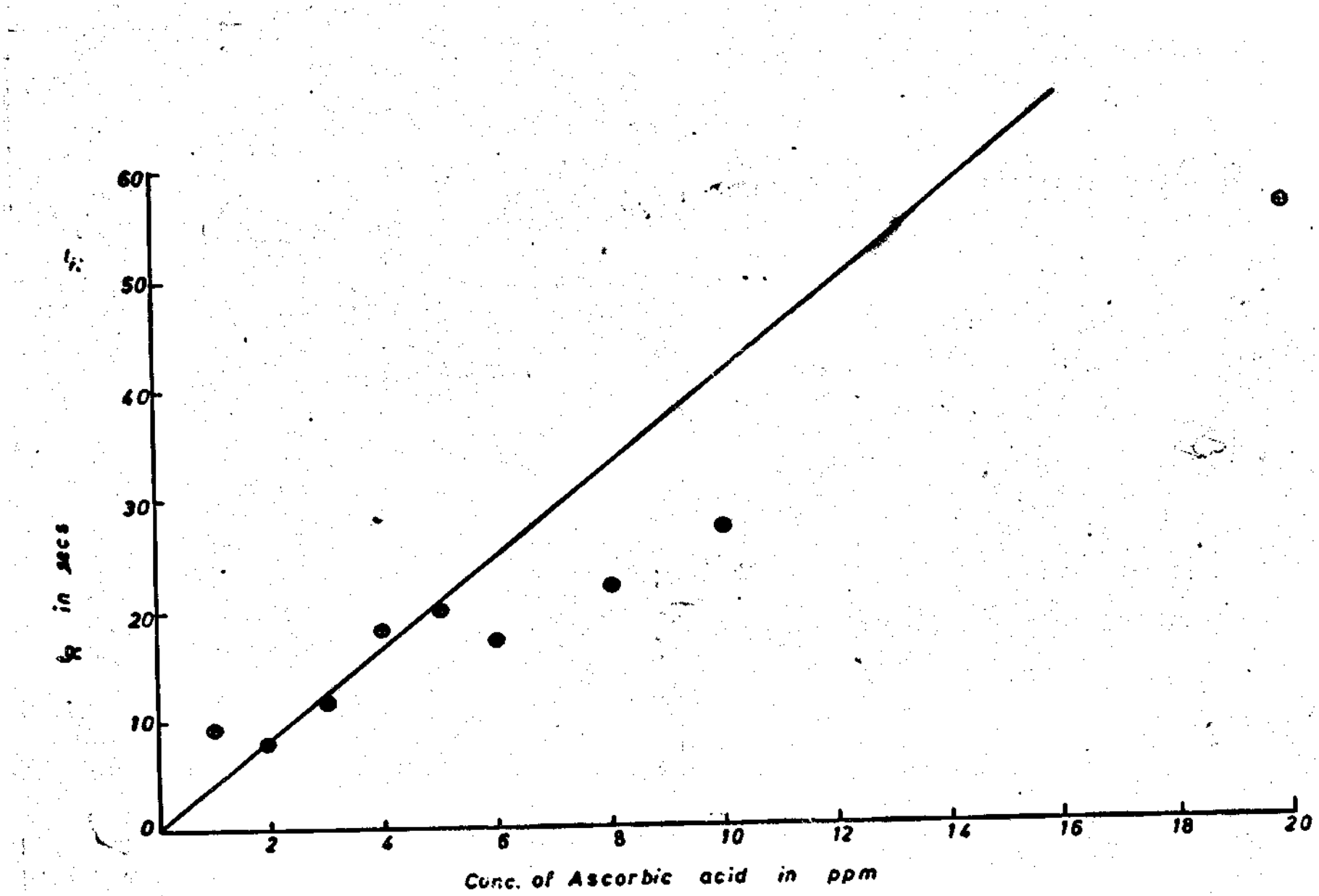


FIG-12: A GRAPH OF $\frac{1}{R}$ Vs ASCORBIC ACID

Since during t_R measurements, the Mo(VI) reactions is eliminated, the t_R value is therefore proportional to the amount of iodine required to react with ascorbic acid in a direct potentiometric titration. Table 4.40 shows that t_R increases as the ascorbic acid concentration is increased. A graphical plot of t_R vs standard ascorbic acid concentration (Fig. 12) gives a straight line in agreement with the above supposition. This curve may be used as a standard curve in a potentiometric titration with ascorbic acid alone. Results obtained from this curve are much higher than those from the kinetics method (Table 4.60).

When competition between iodine and Mo(VI) is induced as during t_c measurements, the time t_c, required to remove all of the ascorbic acid at various concentrations of the ascorbic acid, is fairly constant with minor fluctuations as shown in Table 4.40.

A plot of t_c/t_R vs ascorbic acid concentration (Fig. 11) shows that a straight line is obtained only from zero to 3 ppm ascorbic concentration. This seems to suggest that this method could only be used within the ascorbic acid concentration range of 0 to 3 ppm after which it breaks down due to some other complications in the system which need to be further looked into. It also seems to comply with the initial rate method commonly used in kinetics method.

In a system where there is competition, t_c can never be greater than t_R where there is no competition. It is obvious that t_c/t_R can only have calues of $\leq$ 1. It is in this sense that the intercept of a plot of t_c/t_R vs ascorbic acid concentration on the y-axis can be explained. (Fig. 11).

With respect to the results obtained by the kinetics method (Table 4.60), it may be observed that these are exceptionally high when compared to the results by the other methods. Reasons for this are not immediately clear. Perhaps, it is due to the complex nature of the blood system or from the reduction process. Efforts to identify factors arising from the reduction process proved complicated. There is need for further investigation into this. (See paragraph 4.40).

With respect to the thermal effect, no appreciable change in both the ascorbic acid level and glucose content was observed. The much lower glucose content observed at 24° with 12-14 weeks old chicks in Table 4.60, could be due to experimental errors in diluting the sample solutions.

Studies on guinea pigs (4) women (6) and calves (9) exposed to high environmental temperatures, showed that these animals had lower blood ascorbic acid levels.

This was attributed to an increase in the rate of body metabolism resulting in an increase in the utilization of the vitamin. Man and guinea pigs fall into the category of animals that are unable to synthesize ascorbic acid. On the other hand, rats like chickens are able to synthesize it. A report (14) on rats subjected to high environmental temperatures, showed that ascorbic acid (AA) dehydroascorbic acid (DAA) and diketogulonic acid (DKA) levels in the liver and also the blood serum DAA and DKA levels vary insignicantly under the thermal effect.

Kanfer et al (65) showed that the major metabolic products of l-ascorbic acid metabolism in mammalian tissues are pentonic acids, (L-xylonic and L-Lyxonic acids). With increase in temperature we expect an increase in the rate of body metabolism and therefore the increase in the concentrations of these products. This is an area for further investigation. Chickens are able to synthesize their own ascorbic acid. Therefore, any alteration in the ascorbic acid level would be normalized by the synthesizing process. Thus the constant ascorbic acid level observed with respect to the thermal effect may be explained on this basis.

CONCLUSION

4.30

From the experimental results, the ascorbic acid level of different ages of chickens is unaffected by heat stress within the temperature range of this investigation (18°-41°). At the same time, the glucose content varied insignificantly with respect to the thermal effect. This suggests that the thermal factor has no effect on the ascorbic acid metabolism of chickens under the experimental conditions of this investigation.

It is only possible to conclude from these observations either that

(i) No extra ascorbic acid, above the normal requirement is utilized by chicks within the experimental and temperature conditions.

or (ii) That chicks synthesize ascorbic acid to replace that used in excess of normal requirement.

4.40 Suggestions For Further Study.

1. The Spectrophotometric method (47) might be best applicable to blood and other biological samples if the ascorbic acid is isolated from other reducing substances.

2. The Kinetics method which is still in its infancy, requires that the ascorbic acid be isolated from the blood sample because of the difficulty in suppressing all the interferences in the blood medium. The separation by paper chromatography (52) or some other techniques have been successfully employed.

3. Previous studies show that many factors and substances are related to ascorbic acid. In dealing with a complex system such as blood, a reasonable knowledge of the chemistry of blood and body metabolic processes would be an added advantage

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Appendix

TABLE 1: Results for calibration curve by 2.4-Dinitrophenyl-drazine Reagent.

Micrograms of ascorbic acid in sample	Absorbance			Mean
5	0.100	0.100	0.100	0.100
10	0.155	0.130	0.140	0.142
20	0.320	0.280	0.280	0.293
30	0.485	0.440	0.430	0.452
40	0.685	0.595	0.580	0.587
50	0.750	0.730	0.740	0.730
60	0.950	0.950	0.950	0.950

TABLE 2: Results For Calibration Curve For Glucose Determination by Glucose Oxidase.

mMol/litre	Absorbance			mean
1	0.050	0.050	0.050	0.050
2	0.102	0.100	0.110	0.104
4	0.210	0.192	0.200	0.201
5	0.240	0.240	0.245	0.242
6	0.280	0.255	0.280	0.268
7	0.335	0.330	0.300	0.322
8	0.400	0.400	0.443	0.414
9	0.474	0.450	0.445	0.456
10	0.485	0.500	0.520	0.502

TABLE 3: Colour Development of Iron (11)-1,10-Phenanthroline complex For Ascorbic acid in chicken blood.

Time in minutes	0	30	50	75	90
Absornance	0.03	0.05	0.075	0.085	0.115
Time in Minutes	100	115	125	140	160
Absorbance	0.135	0.150	0.165	0.180	0.200
Time in minutes	180	200	220	270	
Absorbance	0.210	0.210	0.235	0.260	

TABLE 4: Determination of Ascorbic acid in the 30 weeks old chicken. 2, 4-Dinitrophenylhydrazine Re gent.

TABLE 5: Ascorbic Acid Determination at 3.00 p.m.

Identity of bird	Cage Temp. °C	Absrobance		mean	Cage Temp. °C	Absorbance		mean
1	30.5°	0.135	0.140	0.138	26.7°	0.125	0.135	0.130
2	30.5°	0.110	0.120	0.115	26.7°	0.120	0.110	0.115
3	30.5°	0.130	0.115	0.123	26.7°	0.120	0.120	0.120
4	30.5°	0.125	0.100	0.113	26.7°	0.120	0.120	0.120
5	30.5°	0.100	0.110	0.105	26.7°	0.120	0.110	0.115

TABLE 6: Ascorbic Acod Determination at 8.30 a.m.

1	19.5°	0.160	0.160	0.160	18.5°	0.160	0.160	0.160
2	19.5°	0.160	0.165	0.163	18.5°	0.150	0.160	0.155
3	19.5°	0.160	0.150	0.155	18.5°	0.160	0.160	0.160
4	19.5°	0.150	0.150	0.150	18.5°	0.150	0.150	0.150
5	19.5°	0.160	0.160	0.160	18.5°	0.160	0.150	0.155

TABLE 7: Ascorbic Acid Determination After 8hrs Incubation at 30°C.

1	30.0°	0.140	0.140	0.140	30.0°	0.160	0.150	0.155
2	30.0°	0.110	0.105	0.108	30.0°	0.150	0.150	0.150
3	30.0°	0.155	0.150	0.153	30.0°	0.155	0.155	0.155
4	30.0°	0.130	0.140	0.135	30.0°	0.150	0.150	0.150
5	30.0°	0.140	0.150	0.145	30.0°	0.165	0.165	0.165

TABLE 8: Ascorbic Acid Determination. After 8hrs incubation at 37° and 26°C.

Identity of bird	Cage Temp.	Absorbance		mean	Cage Temp.	Absorbance		mean
1	37°	0.145	0.145	0.145	26°	0.100	0.110	0.105
2	37°	0.145	0.135	0.140	26°	0.100	0.110	0.105
3	37°	0.130	0.120	0.125	26°	0.120	0.120	0.120
4	37°	0.140	0.140	0.140	26°	0.115	0.110	0.113
5	37°	0.140	0.150	0.145	26°	0.110	0.100	0.105

TABLE 9: Ascorbic Acid Determination at 42° and 28°

Identity of bird	Cage Temp.	Absorbance		mean	Cage Temp.	Absorbance		mean
1	42°	0.130	0.100	0.120	28°	0.080	0.110	0.099
2	42°	0.135	0.140	0.133	28°	0.090	0.090	0.090
3	42°	0.110	0.110	0.110	28°	0.115	0.105	0.110
4	42°	0.110	0.125	0.123	28°	0.110	0.080	0.099
5	42°	0.115	0.115	0.115	28°	0.110	0.110	0.110

TABLE 10: Glucose Determination at 30.5° and 26.7°

Identity of bird	Cage Temp.	Absorbance		mean	Cage Temp.	Absorbance		mean
1	30.5°	0.05	0.050	0.050	26.7°	0.090	0.085	0.088
2	30.5°	0.05	0.060	0.055	26.7°	0.075	0.075	0.075
3	30.5°	0.06	0.055	0.058	26.7°	0.070	0.070	0.070
4	30.5°	0.04	0.035	0.038	26.7°	0.075	0.080	0.078
5	30.5°	0.06	0.05	0.055	26.7°	0.070	0.080	0.075

TABLE 11: Glucose Determination at 19.5° and 18.5°

Identity of bird	Cage Temp.	Absorbance		mean	Cage Temp.	Absorbance		mean
1	19.5°	0.085	0.080	0.083	18.5°	0.06	0.060	0.060
2	19.5°	0.080	0.070	0.075	18.5°	0.05	0.050	0.050
3	19.5°	0.080	0.080	0.080	18.5°	0.05	0.050	0.050
4	19.5°	0.070	0.070	0.070	18.5°	0.05	0.050	0.050
5	19.5°	0.070	0.060	0.065	19.5°	0.05	0.055	0.053

TABLE 12: Glucose Determination at 30°.

		Absorbance (mean)				Absorbance		(mean)
1	30.0°	0.060	0.075	0.068	30°	0.070	0.080	0.075
2	30.0°	0.070	0.065	0.068	30°	0.070	0.070	0.070
3	30.0°	0.080	0.080	0.080	30°	0.080	0.070	0.075
4	30.0°	0.070	0.110	0.090	30°	0.070	0.075	0.073
5	30.0°	0.075	0.085	0.078	30°	0.075	0.080	0.078

TABLE 13: Glucose Determination at 37°.

		Absorbance		(mean)		Absorbance		(mean)
1	37°	0.075	0.075	0.075	26°	0.070	0.070	0.070
2	37°	0.060	0.055	0.058	26°	0.060	0.060	0.060
3	37°	0.060	0.065	0.063	26°	0.080	0.080	0.080
4	37°	0.060	0.060	0.060	26°	0.070	0.070	0.070
5	37°	0.060	0.060	0.060	26°	0.060	0.060	0.060

TABLE 14: Glucose Determination at 42° and 28°.

Identity of bird	Cage Temp.	Absorbance		mean	Cage Temp.	Absorbance		mean
1	42°	0.080	0.070	0.075	28°	0.070	0.060	0.065
2	42°	0.080	0.085	0.083	28°	0.070	0.070	0.070
3	42°	0.100	0.100	0.100	28°	0.080	0.070	0.075
4	42°	0.090	0.085	0.088	28°	0.070	0.075	0.073
5	42°	0.080	0.075	0.078	28°	0.060	0.060	0.060

TABLE 15: Ascorbic acid levels in 9 weeks old chickens determined by 2,6-dichlorophenolindophenol titration.

IDENTITY OF BIRD	24.50			23.80		
	ml Titration		Mean ml	ml Titration		mean ml
1	2.03	2.30	2.17	1.95	2.00	1.98
2	2.00	2.00	2.00	1.90	2.00	2.00
3	2.00	2.10	2.05	2.30	2.15	2.13
4	2.40	2.30	2.35	2.75	2.70	2.73
5	1.40	1.40	1.40	2.50	2.40	2.45

TABLE 16: Ascorbic acid levels in 11-13 weeks old chickens determined by 2,6-dichlorophenolindophenol titration.

IDENTITY OF BIRD	41°			30°			41°	24°
	ml titration	ml titration	mean	ml titration	ml titration	Mean	ml titration	ml titration
1	1.95	1.50	1.73	1.35	1.50	1.43	1.85	1.68
2	2.05	2.10	2.05	1.60	1.65	1.63	1.75	1.5
3	2.20	2.25	2.23	1.95	1.80	1.88	2.00	1.95
4	2.15	1.60	1.88	2.00	2.00	2.00	1.65	2.15
5	1.85	1.40	1.63	1.35	1.05	1.20	1.50	1.85
6	1.50	1.80	1.65	1.45	1.50	1.48	1.80	2.25
7	1.65	1.60	1.63	1.50	1.50	1.50	1.75	2.40
8	1.85	2.10	1.98	1.90	2.15	2.03	2.00	1.95
9	1.95	1.70	1.83	1.40	1.40	1.40	1.95	2.20
10	2.00	1.95	1.98	2.00	1.85	1.93	2.00	1.90

TABLE 17: Ascorbic acid and Glucose levels of 12-14 weeks old chickens.

IDENTITY OF BIRD	24°				41°			
	Ascorbic acid	Glucose			Ascorbic acid	Glucose		
	Absorbance	Absorbance		mean	Absorbance	Absorbance		mean
1	0.100	0.030	0.030	0.030	0.100	0.075	0.070	0.073
2	0.120	0.030	0.030	0.030	0.130	0.065	0.065	0.065
3	0.125	0.035	0.035	0.033	0.150	0.055	0.065	0.060
4	0.125	0.040	0.040	0.038	0.130	0.065	0.0[illegible]	0.064
5	0.130	0.035	0.035	0.033	0.120	0.065	0.045	0.055
6	0.130	0.040	0.040	0.040	0.150	0.075	0.065	0.070
7	0.110	0.040	0.040	0.038	0.150	0.065	0.065	0.065
8	0.140	0.040	0.040	0.038	0.100	0.065	0.070	0.068
9	0.120	0.040	0.040	0.038	0.075	0.065	0.070	0.068
10	0.100	0.035	0.035	0.035	0.080	0.055	0.055	0.055

TABLE 18: Manual Titration Curves (^{t}R) For Ascorbic acid in blood.

(a) After Reduction with H_2S.

Volume ml(0.00/MI	Potential MV
0.000	271.5
0.039	267.5
0.051	265.5
0.061	265.5
0.109	267.5
0.122	271.5
0.142	278.5
0.181	280.5
0.210	282.5
0.252	290.5
0.275	310.5
0.300	347.5
0.356	397.5
0.388	412.5
0.401	411.5
0.461	425.5
0.502	428.5
0.504	432.5
0.800	434.5
0.907	434.5
1.003	434.5
1.302	434.5

(b) After Reduction with Bacteria.

Volume Ml (0.00/MI	Potential mv
0.000	409.5
0.027	409.5
0.049	409.5
0.086	409.5
0.124	408.5
0.170	408.5
0.230	408.5
0.317	408.5
0.369	407.5
0.430	408.0
0.496	407.5
0.608	406.5
0.810	406.5
1.003	406.0
1.418	404.5
2.033	403.5
2.515	404.0
2.638	404.5
2.676	404.5
2.735	406.5
2.815	410.0
3.026	420.5
3.065	421.5
3.199	422.5
3.242	424.5
3.385	428.5
3.548	431.5

(b) Cont'd

After Reduction with Bacteria

Volume Ml (0.00/MI)	Potential mv
3.788	435.5
3.930	441.5
4.057	442.5
4.511	446.0
4.859	448.0
5.727	449.0
6.622	450.0
7.520	451.0

TABLE 19: Manual Titration curve t_R for ascorbic acid in blood without Reduction.

Volume ml	Potential mv
0.000	385
0.033	385
0.068	385
0.112	380
0.176	379
0.226	378

Table 19.

Volume ml	Potential mv
0.285	376
0.361	375
0.440	371
0.513	369.5
0.581	368.5
0.731	366.5
0.836	364.5
1.001	362.5
1.142	361.5
1.348	360.0
1.505	358.5
1.724	356.0
1.915	354.5
2.060	353.0
2.313	352.0
2.416	350.0
2.514	349.0
2.553	346.0
2.632	345.0
2.709	344.0
2.942	343.0

Volume ml	Potential mv
3.110	342.0
3.349	341.0
3.366	339.0
3.432	336.0
3.638	336.0
4.008	335.5
4.596	335.0
5.342	334.0
6.315	333.0