# THE EFFECTS OF ASCORBIC ACID ON THE IMMUNE SYSTEM OF THE GUINEA PIG (*Cavia porcellus*)

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I, Anne Phelgona Mudiwo, hereby declare that this is my original work and has not been presented for a degree in any other University.

Anne phelgona Mudiwo

10/7/93

This thesis has been submitted with our approval as University supervisors.

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## ABSTRACT

A review of the literature shows that vitamin C has a positive effect on some mechanisms of both natural and acquired resistance against infection. The specific effects of ascorbic acid in these processes have however remained obsecure. The purpose of this study was to investigate the effects of ascorbic acid on the immune system using the guinea pig as a model. The specific objectives were to find out the effects this vitamin on immunoglobulin levels, on T-cell function and on humoral response to viral antigens.

In all the three experiments done, the animals were divided into four groups recievig 0, 0.5, 1.5 and 2.5mg ascorbic acid per 100gm body weight daily. These animals were then maintained on an ascorbic acid deficient diet, and were weighed and bled once every seven days.

In experiment I, the immunoglobulin levels were determined using a `Sandwich' Enzyme Linked Immunosorbent Assay. In experiment II, the T-lymphocytes were cultured and stimulated by mitogens (phytoheamagglutinin and Concanavalin A) after which the response to stimulation was compared. In experiment III, the guinea pigs were innoculated with Hepatitis B surface antigen (HBsAg). The levels of guinea pig anti-HBsAg were determined using a Passive Heamagglutinin test.

The results obtained for experiment I indicated that immunoglobulin levels were significantly higher in the experimental groups than in the levels of the control group, ($p < 0.05$), while the results of experiment II showed no significant difference in the stimulation indexes of all the groups, ($p < 0.05$) and in experiment III, the levels of guinea pig anti-HBsAg increased with increase in ascorbic acid dose.

It was therefore concluded that ascorbic acid is involved in the production of immunoglobulins, that the vitamin enhances the humoral response against viral antigens and that it does not seem to enhance T-cell proliferation following mitogenic stimulation.

From the results of this study the following areas have been suggested for further investigation.

1. The role of ascorbic acid in enhancing the production of immunoglobulins against specific antigens.
2. The phase of humoral response that the vitamin is involved in.

# CHAPTER ONE

## 1.0 INTRODUCTION AND LITERATURE REVIEW.

## 1.1 INTRODUCTION

For many years since its discovery towards the end of the last century, ascorbic acid (vitamin C) has been known to have therapeutic effects. Its mode of action or the systems it affects have however remained unclear. While several studies have been carried out by scientists and nutritionists over the past 90 years, looking at various possible activities of this vitamin, its therapeutic role in viral infections has received special attention. The results of such studies, some of which are discussed in the next section, have only served to point out to the diversity of the physiological effects of ascorbic acid in different species. Due mainly to this diversity of effects, there have been numerous statements and suggestions on the mode of action and therapeutic value of vitamin C, but most of these are yet to be proven.

### 1.1.1 History of Ascorbic Acid

It is known that early travellers particularly sailors suffered from scurvy, a nutritional disease caused by a severe deficiency of ascorbic acid. Those affected were crusaders of the thirteenth century and the seamen of the fourteenth century. In Vasco da Gamma's

voyage round the Cape of Good Hope, two thirds of the crew died of scurvy (Wilson *et al.*, 1965). In 1947, James Lind, a ship's surgeon in the English navy had the genius to observe, record and experiment with scurvy patients and showed that scurvy could be cured by orange and lemon juices (Wilson *et al.*, 1965; Bharaj, 1977). While citrus and other fresh fruits had long been known to have preventive and curative effects on scurvy, the scientific basis for the study of ascorbic acid was first initiated in 1907 when scurvy was proved experimentally in guinea-pigs. The acid was then isolated in 1928 from orange and cabbage (Proudfit & Robinson, 1955).

### 1.1.2 Evolution of ascorbic acid

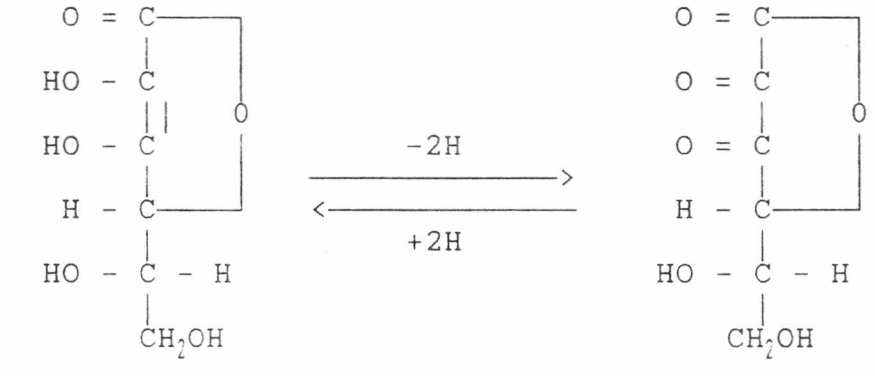
The loss of capacity to synthesize ascorbic acid is an important example of evolutionary loss. Most animals can synthesize their own supply of ascorbic acid; the amphibians and reptiles synthesize ascorbic acid in the kidneys whereas in mammals it is synthesized in the liver. This biosynthetic capacity is lost in the guinea pig and such highly evolved mammals as Indian fruit eating bat, monkeys, man and also the Bulbul bird (Burton, 1976; Bharaj, 1977). This inability is due to a common genetic defect caused by the absence of the gene that codes for the synthesis of the enzyme

gulono-lactone-oxidase. The mutation leading to the loss of this gene was neutral and not lethal (Kings & Jukes, 1969), i.e the mutants did not become extinct as the enviroment continued to furnish the adequate amount of ascorbic acid. The significant dietary sources of ascorbic acid are friuts, vegetables and liver (Wohl & Goodhard, 1960).

The animals that synthesize ascorbic acid either in the liver or kidneys use D-glucose as a precursor (Burns & Evans, 1956). The D-glucose then passes through stages namely D-glucoronolactone and L-gulonolactone before becoming L-ascorbic acid (Burns, 1957). The animals that are not able to synthesize the vitamin, lack two enzymes required in this process. These are L-gulono oxidase and D-glucorono reductase which convert D-glucoronolactone to L-gulonolactone (Chatterjee *et al.*, 1961). Due to this inability to synthesize ascorbic acid, guinea pigs require a dietary C supply of vitamin C. A daily supply of 0.2g/kg is low but it is anti scorbutic (Bendich *et al.*, 1984). The maximum growth rate is obtained with a dose of 2g/kg body weight (Nandi *et al.*, 1973). The dosage should not exceed a particular limit depending on the kind of diet the animals are fed on. A daily dose of 50mg or more is toxic to guinea pigs fed on unfortified wheat diet but it is not dangerous as long as the food consumed is nutritionally balanced (Nandi *et al.*, 1973).

## 1.1.3. Properties of ascorbic acid

Ascorbic acid (vitamin C) is a water soluble crystaline compound which is also soluble in ethyl alcohol and glycerol (Burton, 1976; Pike & Brown, 1975). Though the vitamin is quite stable in the dry state, it is easily oxidized in solution (Pike & Brown, 1975). Ascorbic acid also undergoes destruction when exposed to air, heat, light or metals such as copper and iron (Wilson *et al.*, 1965). Ascorbic acid has a melting point of 192°C and its emperical formular is $C_6H_8O_6$ with a molecular weight of 176. It is easily oxidized to form dehydroascorbic acid, which is also easily reduced back to the original form (Pike & Brown, 1975; Burton, 1976). Ascorbic acid therefore has a high oxidation-redution potential and the redox system is presented as follows:




Ascorbic Acid  Dehydroascorbic Acid

Ascorbic acid is directly absorbed from the small intestines by simple diffusion. Through tissue saturation, the human adult is able to store sufficient amounts of the vitamin although it is metabolised partly to oxalic acid some of which is excreted in urine (Briggs *et al.*, 1973).

Ascorbic acid is required for the formation of connective tissue and synthesis of collagen (Shimizu *et al.*, 1965) in which case it acts as an electron donor in hydroxylation of proline (Bharaj, 1977). It is essential for the functional intergrity of the sulfhydryl group of enzymes. Ascorbic acid influences the formation of heamoglobin, erythrocyte maturation, certain immunological reactions of the body and also functions in protecting the body against infectious diseases and bacterial toxins (Satoskar & Bhadakor, 1980).

## 1.2. LITERATURE REVIEW

Ascorbic acid has been considered as important only in the prevention and cure of scurvy. However it has been shown through various experiments that ascorbic acid not only has a direct influence on the immune system (Long, 1950), but also a positive effect on some mechanisms of natural resistance against infection (Banic, 1982). This is supported by the high concentration of ascorbic acid in leucocytes and its

mechanisms of natural resistance against infection (Banic, 1982). This is supported by the high concentration of ascorbic acid in leucocytes and its rapid expenditure during infection and phagocytosis (Thomas & Holt, 1978). Evidence published to date shows an involvement in the leucocyte migration and in phagocytosis as well as in induction and expression of delayed hypersenstivity (Thomas & Holt, 1978). The other areas influenced by ascorbic acid include T-cell function (Anthony *et al.*, 1979) and the complement system (Fraser *et al.*, 1980) and production of interferons (Siegel & Mortorn, 1977),

### 1.2.1 **Ascorbic acid in leucocytes.**

In humans ascorbic acid is found in high concentrations in blood leucocytes (King, 1968). The predominant cell type in the leucocyte population is the polymorphonuclear leucocyte which attains ascorbic acid levels of 1.0µg/mg protein (DeChatelet *et al.*, 1974). Mononuclear phagocytes contain even higher concentrations of ascorbic acid (2.0µg/mg protein) with both peritoneal and alveollar macrophages being rich in ascorbate (Thomas & Holt, 1978). In the B and T lymphocytes ascorbic acid occurs in millimolar concentrations (Bergesten *et al.*, 1990).

That ascorbic acid is important in luecocyte function is suggested by the finding that the level of this vitamin rapidly decreases following viral infection and returns to normal after recovery. It has also been shown that there is active uptake of ascorbic acid by leucocytes in subjects with colds following loading doses of the vitamin and that the leucocytes absorb large doses of the vitamin when they migrate into an infected area (Thomas & Holt, 1978).

Leucocyte ascorbic acid levels have been shown to decrease in cases associated with immunological suppression for example pregnancy is associated with depressed cellular immunity and pregnant women exhibit depressed levels of leucocyte ascorbic acid, steroid therapy which produces severe immunosuppression decreases leucocyte ascorbic acid (Thomas & Holt, 1978). These results therefore suggest that ascorbic acid influences certain processes of the immune system, some of which are discussed below.

### 1.2.2 Ascorbic acid and Phagocytosis

The ability of phagocytic cells to ingest and destroy bacteria has been associated with the presence of adequate ascorbic acid in these cells such that cells from individuals with low levels of ascorbic acid display

low phagocytic activity (Lewin, 1976). Furthermore, in such vitamin C deficient individuals, the bactericidal action of the phagocyte is impaired (Bach, 1976). Experiments with guinea pigs performed over a period of four weeks showed that increasing doses of ascorbic acid resulted in a significantly increased number of ingested bacteria per phagocyte (Lewin, 1976). Ascorbic acid deficient guinea pigs yield fewer macrophages which are smaller in size and show reduced migration (Chandra, 1980).

Furthermore, in humans, ascorbic acid occurs in millimolar concentrations in monocytes which suggests that the vitamin does play a role in monocyte function, (Bergesten *et al.*, 1990). In the human polymorphonuclear leucocytes, ascorbic acid is found in micromolar concentrations which has led to the suggestion that the vitamin may play a role in the promotion of oxidative destruction of micro-organisms, the preservation of neutrophil intergrity and the protection of host tissues by inactivating free radicals and oxidants (Washko *et al.*, 1990).

Goetzl *et al.*, (1974), using tissue concentration *in vitro* found that ascorbic acid increased random migration of neutrophils, as well as migration induced by the complement component C5a. Neutrophils pre-exposed to chemotactic factors become inactivated in such a way that

they are unresponsive to chemotactic stimulation (Ward & Becker, 1968). Such deactivated neutrophils however maintain an intact random migration (Patrone *et al.*, 1980). In an experiment where graded amounts of ascorbic acid were administered, the loss of chemotactic responsiveness of deactivated neutrophils was prevented by appropriate concentrations of the vitamin (Patrone *et al.*, 1980).

### 1.2.3. Ascorbic acid and Hypersensitivity

Some evidence indicates that ascorbic acid may play a role in immediate hypersensitivity reactions (Thomas & Holt, 1978). Mast cells contain extremely high levels of ascorbic acid, approximately three times as much as macrophages (Thomas & Holt, 1978).

Reduced release of histamine and slow-reacting substance of anaphylaxis was observed in sensitized lung fragments of guinea pigs that were ascorbic acid deficient, and dietary replenishment of ascorbic acid restored normal *in vitro* responsiveness (Panush & Delafuente, 1985). Kumar and Axelrod (1969), compared the capacity of scorbutic and normal guinea pigs with similar titres of antibody to diptheria toxoid, to mount Arthus-type reactions following challenge with this antigen and they found depressed skin reactions in the scorbutic group. However, Panush and Delafuente (1985)

also found similar reduction in the reaction of scorbutic guinea pigs to the non-specific irritants, histamine-provoked bronchoconstriction in humans and in guinea pigs. Ascorbic acid administration proved effective in reducing mortality and prolonging the survival of mice induced with anaphylaxis. Animals on a vitamin C regimen showed lower tissue histamine levels, suggesting a protective effect of vitamin due to lowering of histamine concentrations. Conceivably, vitamin C could be of benefit in the treatment of immediate-type hypersensitvity such as asthma, hayfever. and perhaps other situations where histamine levels are elavated (Siegel & Leibovitz, 1982).

Ascorbic acid has also been indicated in delayed-type hypersensitivity. This was first observed by Mueller and Kies (1962) when they demonstrated depressed responses to the mycobacteria in Complete Freund's adjuvant in scorbutic guinea pigs and observed the reverse following ascorbate supplementation. Scorbutic guinea pigs anergic to mycobacteria possesed sensitized lymphocytes that could transfer delayed-type hypersensitivity to normal animals. In contrast, normal animals were ineffective in transfering delayed-type hypersensitivity to scorbutic recipients (Thomas & Holt, 1978). Scorbutic animals show a depressed inflammatory response to a non-specific

irritant. This could be due to a defect in migration to recruited cells to the site of challenge rather than a central defect in the lymphocyte function (Thomas & Holt, 1978; Panush & Delafuente, 1987).

### 1.2.4. Ascorbic acid and Antibody Production.

Long (1950) reported that the addition of ascorbic acid to immunizing doses of antigen appeared to increase antibody production and deprivation apparently reduced the response.He also. He also claimed that while scorbutic guinea pigs mounted normal primary responses, their secondary responses were depressed thirty-fold. However these results were challenged by Kumar & Axelrod (1969) who repeated the study which involved measuring primary and secondary responses to diptheria toxoid in normal and scorbutic guinea-pigs and they failed to detect any deficiency in either the primary or the secondary responses of the guinea-pigs. In another experiment which was carried out in a double blind study, volunteers received placebo or 1g or 3g of vitamin C for seven days, the results showed no significant differences among the groups although IgM levels did increase with the increasing dose of ascorbic acid (Panush *et al.*, 1982).

Anderson & Van (1980) also carried out an experiment to investigate the effect of ascorbic acid and the results showed no significant difference between the groups.

However there are experiments that have shown positive effects of vitamin C on antibody production. When two groups of guinea pigs, one receiving ascorbic acid and the other receiving none were injected with sheep Red Blood Cells (SRBC), the peaks of antibody titres were significantly greater and occurred earlier in those given ascorbic acid (Prinz *et al.*, 1980). Similarly in another experiment guinea-pigs divided into three groups and receiving 0mg, 25mg, and 250mg of ascorbic acid daily were injected with Bovine Serum Albumin (BSA). The anti-BSA immunoglobulin levels on day 14 were 0.53, 0.89, and 0.73 while on day 28 the levels were 2.24, 3.55 and 2.72mg antibody per millitre respectively, (Fraser *et al.*, 1980).
However the effect of ascorbic acid in antibody production has remained a matter of dispute.

### 1.2.5. Ascorbic Acid and Infections.

An important function for leucocyte ascorbic acid is suggested by the finding that levels of this vitamin rapidly decrease during a viral infection and return to normal after recovery (Fraser *et al.*, 1980). The immune systems' bactericidal and viricidal effects have been

found to be sensitive to leucocyte ascorbic acid levels. This was observed in an experiment where the bactericidal action of the phagocyte was impaired in vitamin C deficiency (Bach, 1976; Bendich, 1987). Ascorbic acid has also been shown to influence prognosis in various viral infections (Briggs, 1984). Numerous controlled clinical trials have been conducted to study the effect of vitamin C in preventing or treating colds, consequently there is a general agreement that ascorbic acid supplementation does not alter or decrease the incidence of the cold (Anderson *et al.*, 1972; Anderson *et al.* 1975; Coulehan *et al.*, 1976; Ludvigsson *et al.*, 1977). However, several studies have shown that the duration and severity of a cold is reduced by ascorbic acid supplementation (Briggs, 1984; Baird *et al.*, 1979; Pitt & Costrini, 1979; Carr, 1981). Mink *et al.*, (1987) found out that the severity of the signs and symptoms were much lower in ascorbic acid supplemented group and twice as severe in the placebo group. Elwood *et al.*, (1977) also showed that ascorbic acid has a significant therapeutic effect on the common cold, in their experiment the duration of sickness was almost two days less in supplemented group than the placebo group. In another study made on natural orange juice, synthetic orange juice and a placebo in the prevention of the common cold, the natural and synthetic orange juices

showed 14% to 21% reduction in total symptoms due to common cold (Baird *et al.*, 1979).

Vitamin C has also been implicated in other infections. Briggs,(1984) observed that its requirements are increased during active tuberculosis in which the tissue levels of ascorbic acid are reduced while deficient animals are more susceptible to infection. The author also observed that during whooping cough infection, there depletion of tissue ascorbic acid, however high doses of ascorbic acid do not improve the course of the disease nor reduce the incidence of complications; and that controlled studies of ascorbic acid in the various forms of hepatitis suggest that high doses of the vitamin are unable to reduce the incidence of posttransfusion hepatitis.

Ascorbic acid also plays a role in bacterial infections. Bogert *et al.*, (1966) found out that animals receiving lower intakes of ascorbic acid suffered greater injury from doses of Diptheria. They also showed that guinea pigs on limited vitamin C intake succumbed to innoculation with strains of bacteria which had no effect on control animals.

Although a number of workers have published observations and data that suggest a relationship between ascorbic acid and infectious diseases, the general opinion has been that such diseases merely reduce

(non-specifically) the concentration of vitamin C in tissues. A high vitamin C intake seems to be of little importance in the treatment of infectious diseases.

### 1.2.6. Role of ascorbic acid in T-cell function and cell mediated Immunity.

Ascorbic acid is found in millimolar concentrations in T-cells. Though not clear, its role may be associated with any of the T-lymphocyte functions such as recognition of antigenic epitopes and the biosynthesis of lymphokines (Bergsten *et al.*, 1990). Some deleterious effects of vitamin C deficiency on cell mediated immunity have been observed (Chandra & Newberne, 1977). Mueller and Kies (1962) observed reduced responsiveness to the mycobacteria of Complete Freund's Adjuvant in scorbutic guinea pigs and further showed that the condition could be reversed by dietary ascorbate supplementation. Further reports say that experimental allergic encephalomyelitis was induced less well in scorbutic animals than in normal guinea pigs and it was noted that lymphocytes from anergic scorbutic guinea pigs could transfer delayed-type hypersensitivity to normal animals while lymphocytes from the normal animals could not transfer immunity to scorbutic hosts (Thomas & Holt, 1978; Panush & Delafuente, 1985). Furthermore, a reduced inflammatory response of scorbutic animals to non

specific irritants has also been observed (Thomas & Holt, 1978). It was suggested that impaired cellular responses in scorbutic animals may reflect, either defects in the micro-vasculature permeating localization of reactive cells or dysfunction of cell migration (Panush & Delafuente, 1985). Anthony *et al.*, (1979) also suggested that a defect in cell mediated cytotoxicity in scorbutic guinea pigs is a reflection in dysfunction of a cell type (T-lympocyte) to mediate cytotoxicity. Although studies have been carried out to find out the effect of ascorbic acid on cell responsiveness to mitogens, the results have been contradictory. Some investigators have observed inhibitions in *in vitro* cell responsiveness to mitogens by vitamin C (Munster *et al.*, 1977; Ramirez *et al.*, 1980). Others have found no effect whatsoever (Anderson *et al.*, 1979), while others have documented enhanced responsiveness by vitamin C (Delafuente & Panush, 1980; Panush & Delafuente, 1979).

From the above observations, it is clear that there have been sporadic studies on the role of vitamin C in the prevention of diseases. There is need therefore to carry out further investigations to find out exactly what physiological systems are boosted by this vitamin.

## 1.3. Justification

The importance of ascorbic acid as an essential nutrient is well known; but its mode of action still remains speculative in biological systems. It is generally considered to have only two functions in the body namely prevention and treatment of scurvy. The vitamin has also been implicated in the immune system and in the prevention and cure of viral infections such as the common cold.

Several experiments have been conducted as cited above, which have shown that the vitamin has a direct influence on the immune system but the nature of the influence is still obscure. Though ascorbic acid is found in millimolar concentrations in B and T cells, its function in these cells is not clear.

There is therefore need to carry out further studies in order to establish the immunological mechanisms that are enhanced,impeded or inhibited by the presence of this vitamin. Such studies could then form the basis for more work on the therapeutic levels of ascorbic acid with the view to improving its application in antiviral treatment.

## 1.4 Aim and Objectives

The general aim of this study was to investigate the effect of ascorbic acid on the immune system of the guinea pig, with the following specific objectives:

1. To determine the influence of ascorbic acid on B-cell function.

2. To determine the influence of ascorbic acid on T-lympocyte function.

3. To determine the effect of ascorbic acid on the humoral response to a viral surface antigen.

# CHAPTER TWO

## 2.0 MATERIALS AND METHODS.

### 2.1 Materials

(a) Guinea pigs.

Guinea pigs were used in all the experiments because they readily become scorbutic when fed on an ascorbic acid deficient diet. The animals were obtained from National Public Health Laboratories and International Laboratory for Research on Animal Diseases, (ILRAD). The guinea-pigs were maintained on an ascorbic acid deficient diet and water throughout the experimental period. Groups of these animals were maintained on specified doses of vitamin C orally during the entire period for each experiment. The guinea pigs were weighed and bled once every seven days.

(b) Rabbits

New Zealand white rabbits were obtained from the Zoology Department University of Nairobi and were maintained on Rabbit pellets, vegetables, and water during the experiment. These were used in the production of anti-sera to guinea pig immunoglobulins.

(d) Ascorbic Acid.

The Vitamin was obtained from Roche pharmaceuticals in powder form and was reconstituted in distilled water

to appropriate concentration for the specific groups as indicated below:

| GROUP: | DOSE:(mg/100gmbodyweight/day) |
|---|---|
| A | 0.0 |
| B | 0.5 |
| C | 1.5 |
| D | 2.5 |

Fresh solutions were made every day and administered orally using a syringe and a blunt needle.

(d) Ascorbic acid deficient diet

A diet containing all the other nutrients except ascorbic acid was obtained from TAMFEEDS (K) and animals were fed ***ad libitum.*** The composition of the feeds are shown in appendix III.

2.2 Methods.

2.2.1 Experiment I: Effect of Ascorbic Acid on immunoglobulin levels

32 guinea pigs were divided into four groups (A,B,C and D) of eight animals each. Group A was designated the control group and thus received no ascorbic acid,

while groups B, C and D recieved 0.5mg, 1.5mg and 2.5mg of ascorbic acid per 100gm body weight per day respectively. The recomended daily dose of ascorbic acid for guinea pigs is 2mg/100gm body weight and this was used to determine the doses above. This experiment was aimed at establishing the effect of ascorbic acid on immunoglobulin levels.

During the experiment the guinea pigs were kept in pairs in plastic cages and the woodshaving was replaced twice every week. Each animal was weighed on day 0 of the experiment and then once every seven days until the end of the experiment. They were all maintained on the ascorbic acid deficient diet, and their respective amounts of ascorbic acid which was administered by holding the animal with its belly facing upwards and the solution then dispensed into its mouth ensuring that all the suspension was swallowed (see plate II).

Each animal was bled via cardiac puncture (see plate I) on days 0, 7, 14, 21, and 28. The blood was allowed to clot at room temperature and then keft overnight at $4^{0}C$. The next day the serum was pippeted out and centrifuged at 1000g for five minutes and the serum samples were then stored at $-70^{0}C$ untill they were tested for immunoglobulin levels.

Immunoglobulin levels were determined by the

Sandwich Enzyme Linked Immunosorbent Assay (ELISA). Flat-bottomed 96 well microtitre plates were coated overnight with 100µl Goat anti-guinea pig IgG (Whole molecule; Sigma, USA) diluted in carbonate-bicarbonate buffer (pH, 9.6) to a protein concentration of 20µg/ml. The plates were then washed three times in PBS Tween (see appendix II), every wash taking one minute. After washing, 100µl of the serum samples were added to columns 2 to 11 (see fig. I) The plates were then covered and incubated for 1 hour in humid conditions at $37^{0}$C. After incubation, the plates were washed as before, after which 100µl of the conjugate (Anti-guinea pig IgG Peroxidase conjugate; Sigma, USA) was added to columns 2 to 11 and then incubated as before. The plates were then washed and 100µl of the substrate (ABTS-peroxidase substrate; KPL, USA) was added to each well and incubated for 30 minutes at room temperature. After incubation the optical densities were read using a Multistan ELISA reader (Flow Laboratories) at 492nm.

| A1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| B | | | | | | | | | | | |
| C | | | | | | | | | | | |
| D | | | | | | | | | | | |
| E | | | | | | | | | | | |
| F | | | | | | | | | | | |
| G | | | | | | | | | | | |
| H | | | | | | | | | | | |

*Figure I: Microtitre plate arrangement for the ELISA test.*

**KEY:**

**Column 1:** **Substrate control**

**Column 2 to 11** **Goat anti-guinea pig IgG, samples, substrate and conjugate.**

**Column 12** **Substrate and conjugate**

*Plate I: Oral administration of ascorbic acid.*

*Plate II: Bleeding by cardiac puncture.*

### 2.2.2 Experiment II: Analysis of T-cell function

Twenty guinea pigs were divided into four groups and labelled as in section 2.2.1. These were bled by cardiac puncture using syringes precoated with heparin. A quantity of 2mls of blood was then layered on 3mls of lymphoprep (seperation media) in 15ml centrifuge tubes (Falcon) without predilution. This was centrifuged at 500g for 25 minutes. The resultant band of cells at the interface of plasma and lymphoprep was removed using a pasteur pipette, and then washed two times in incomplete RPMI 1640 wash medium (see appendix II) by centrifuging at 650g for ten minutes after which the cells were suspended in 1ml complete RPMI 1640 culture medium. The cells were then counted using a heamocytometer and then adjusted to a concentration of 1.2 $x10^6$/ml before stimulation with two mitogens namely phytohaemagglutinin (PHA) and concanavalin A (con A). The cells were cultured in the U-shaped, 96 well tissue culture plates (Flow Laboratories). The first four wells of every row acted as controls without mitogen, the next four had 10µl of PHA and the last four had 10µl of Con A. After addition of the mitogens, 100µls of the cell suspension was added to every well. The plates were then incubated at $37^0$C in a humidified incubator with 5% $CO_2$ in air gas phase for three days (72 hours). After 72 hours the plates were pulsed with 5µci by adding $H^3$-thymidine to every well followed by another

incubation under the same conditions for 18 hours. The cells were then harvested on glass fibre filter paper using a multichannel cell harvester (Skatron, USA). The paper was left to dry overnight at room temperature. The next day each disc of the filter paper was placed in a scintillation vial containing 1ml of scintillation fliud and then β-emmissions counted on the β-counter.

| A1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| B | | | | | | | | | | | |
| C | | | | | | | | | | | |
| D | | | | | | | | | | | |
| E | | | | | | | | | | | |
| F | | | | | | | | | | | |
| G | | | | | | | | | | | |
| H | | | | | | | | | | | |

***Figure** II: Microtitre plate arrangament for the cell culture.*

KEY:

Column 1 to 4: Control

Column 5 to 8: PHA

Column 9 to 12: Con A

**2.2.3 Experiment III: Effect of ascorbic acid on humoral response to HBsAg.**

Twenty animals were divided into four groups of five animals each and labelled. Group A was a control group while groups B, C and D recieved 0.5mg, 1.5mg and 2.5mg of ascorbic acid per 100gm of body weight per day respectively. During the experiment the animals were kept in cages made of wire mesh and they were fed on the ascorbic acid deficient diet *ad libitum*, water and the appropriate dose of ascorbic acid. They were weighed once every seven days which was useful in the calculations of the amount of ascorbic acid required by every animal (see appendix I). The vitamin was reconstituted in distilled water every morning and administered as described in section 2.2.1.

All the animals were innoculated with 0.5mg of HBsAg emulsified in Complete Freunds' Adjuvant on day O and boosted on days 7 and 21 of the experiment. Innoculation was done on the foot pad (see plate III) because guinea pigs have been found to respond best to HBsAg through this route (Kaiguri *et al.*, 1990).

The animals were bled on days 0, 7, 14, 21 and 28 as described in section 2.2.I. The blood was left at room temprature overnight and then centrifuged at 957.6g for five minutes in order to obtain the serum samples which were used

in determination of guinea pig anti-HBsAg antibodies using Passive Heamagglutinin test. V-shaped 96 well plates (Microtech, Japan) were used in this test. To each well was added 25µl of Passive Haemagglutinin buffer (see appendix II) followed by 25µl of the test samples using microdiluters. The latter were added to the first well of every row except the last two rows which were reserved for positive and cell controls. The samples were then double diluted to the end of the plate. After dilution, 25µl of the Passive haemagglutinin cells (fixed Sheep Red Blood Cells coated with Purified HBsAg) were added to all the wells, then mixed for 30 seconds using a shaker and then incubated for 2 hours after which the results were read.

Plate III: *Guinea pig being innoculated with HBsAg via the foot pad.*

### 2.2.4 Analysis of data

The data in section 3.1 which shows the optical densities at 492nm was analysed by Kruslal-Wallis statistical method.

In the experiment on analysis of T-Cell function, the stimulation indexes were calculted by dividing the readings of the mitogen stimulated samples by the readings of the control samples

$$\text{i.e} \quad \frac{\text{Mitogen}}{\text{Control}}$$

The medians of the stimulation indexes were then calculated and used in the Kruskal-Wallis statistical method.

The data which shows the levels of guinea pig anti-HBsAg given in ng/ml (section 3.3) was analysed by regression statistical method.

An attempt was made to investigate the effect of ascorbic acid on recovery from irradiation, all the animals (12) were exposed to total body irradiation at a dose of 250 rads. They were then divided into two groups of six each, group A being the control group i.e. receiving no asorbic acid while group B recieved 2.5mg ascorbic acid per 100g body weight per day. They were maintained on the ascorbic acid deficient diet and water and were bled on days 0 and 7. Unfortunately, All the animals died after the second bleed and it was not possible to repeat this experiment due to logistical problems.

# CHAPTER THREE

## 3.0 RESULTS

### 3.1 Effect of ascorbic acid on Immunoglobulin levels.

The levels of IgG in the three ascorbic acid dosage groups B, C and D were significantly higher than those of the control group A, ($P < 0.05$; see table I). For instance, on day 14 the readings for dosage groups B, C, & D were 0.117, 0.176 and 0.143 absorbance at 492nm respectively while that of control group A was 0.084 (see table I). However, there was no significant difference in the levels amongst the three dosage groups e.g on day 28 the readings for B, C & D were 0.169, 0.197 & 0.145 absorbance at 492nm respectively. This indicates that in the presence of ascorbic acid the production of the IgG is enhanced but the dose does not seem to matter.

| | | Mean OD (492 nM) | | | |
|---|---|---|---|---|---|
| Group | | A | B | C | D |
| | 0 | 0.057 | 0.130 | 0.144 | 0.121 |
| D | 7 | 0.055 | 0.110 | 0.158 | 0.110 |
| A | 14 | 0.084 | 0.117 | 0.176 | 0.143 |
| Y | 21 | 0.093 | 0.151 | 0.129 | 0.155 |
| | 28 | 0.040 | 0.169 | 0.167 | 0.145 |

*Table I: Mean Optical density values at 492nM reflecting the levels of serum IgG in all the groups over a period of 28 days. The ascorbic acid dosage groups (0.05, 1.5, 2.5mg/100gm) IgG levels were significantly higher than the control group, $P < 0.05$*

## 3.2 Analysis of T-Cell function.

The results in tables II & III show that ascorbic acid did not influence T-lymphocyte proliferation because there was no significant difference in the stimulation indexes of all the different groups, ($P > 0.05$) stimulated by the two mitogens( PHA and Con A) For instance on day 14 the PHA stimulation indexes were 4.4, 4.2, 3.1, & 2.7 and Con A stimulation indexes were 3.7, 3.7, 5.6 & 2.6 for groups A, B, C & D respectively. The stimulation indexes did not change with increase in time either; for instance the consecutive PHA stimulation indexes of days 7 and 14 for groups A, B, C & D were 7.0, 4.7, 3.2 & 3.3 and 4.4, 4.2, 3.1 & 2.7 respectively; while Con A stimulation indexes were 2.6, 1.5, 2.4 & 4.8 and 3.7, 3.7, 5.6 & 2.6 respectively. (see tables II and III).

| Group | | Stimulation Index A | B | C | D |
|---|---|---|---|---|---|
| | 0 | 3.1 | 3.2 | 1.6 | 1.6 |
| D | 7 | 7.0 | 4.7 | 3.2 | 3.3 |
| A | 14 | 4.4 | 4.2 | 3.1 | 2.7 |
| Y | 21 | 14.2 | 7.8 | 4.3 | 2.1 |
| | 28 | 1.6 | 15.3 | 24.8 | 5.9 |

***Table II: Medians of the PHA stimulation indexes for groups of animals given different doses of ascorbic acid.*** *P > 0.05.*

| Group | | Stimulation Index A | B | C | D |
|---|---|---|---|---|---|
| | 0 | 1.0 | 1.6 | 0.9 | 0.7 |
| D | 7 | 2.6 | 1.5 | 2.4 | 4.8 |
| A | 14 | 3.7 | 3.7 | 5.6 | 2.6 |
| Y | 21 | 10.1 | 4.9 | 2.2 | 1.8 |
| | 28 | 2.1 | 22.4 | 5.9 | 5.1 |

*Table III: Medians of Con A stimulation indexes for groups of animals given different doses of ascorbic acid.* No *significant difference, P > 0.05.*

### 3.3 Effect of ascorbic acid on humoral response to HBsAg.

The levels of guinea pig anti-HBsAg antibodies and their rates of change were monitored. The antibody levels of the groups were significantly different ($P < 0.05$) i.e the levels increased with increase in ascorbic acid dosage, for example on day 28 the antibody levels were 283.4ng/ml, 512.4ng/ml, 682.2ng/ml and 1554.7ng/ml for groups A, B, C and D respectively. The significant increase in antibody levels was mainly observed after the first boosting which was on day 7; uptill day 7, the levels were not consistent with ascorbic acid dose, for example on day 7, the antibody levels were 0.84ng/ml, 0.36ng/ml, 0.87ng/ml and 0.42ng/ml for groups A, B, C and D repectively (see fig III). The results indicate that ascorbic acid enhances humoral response in guinea pigs.

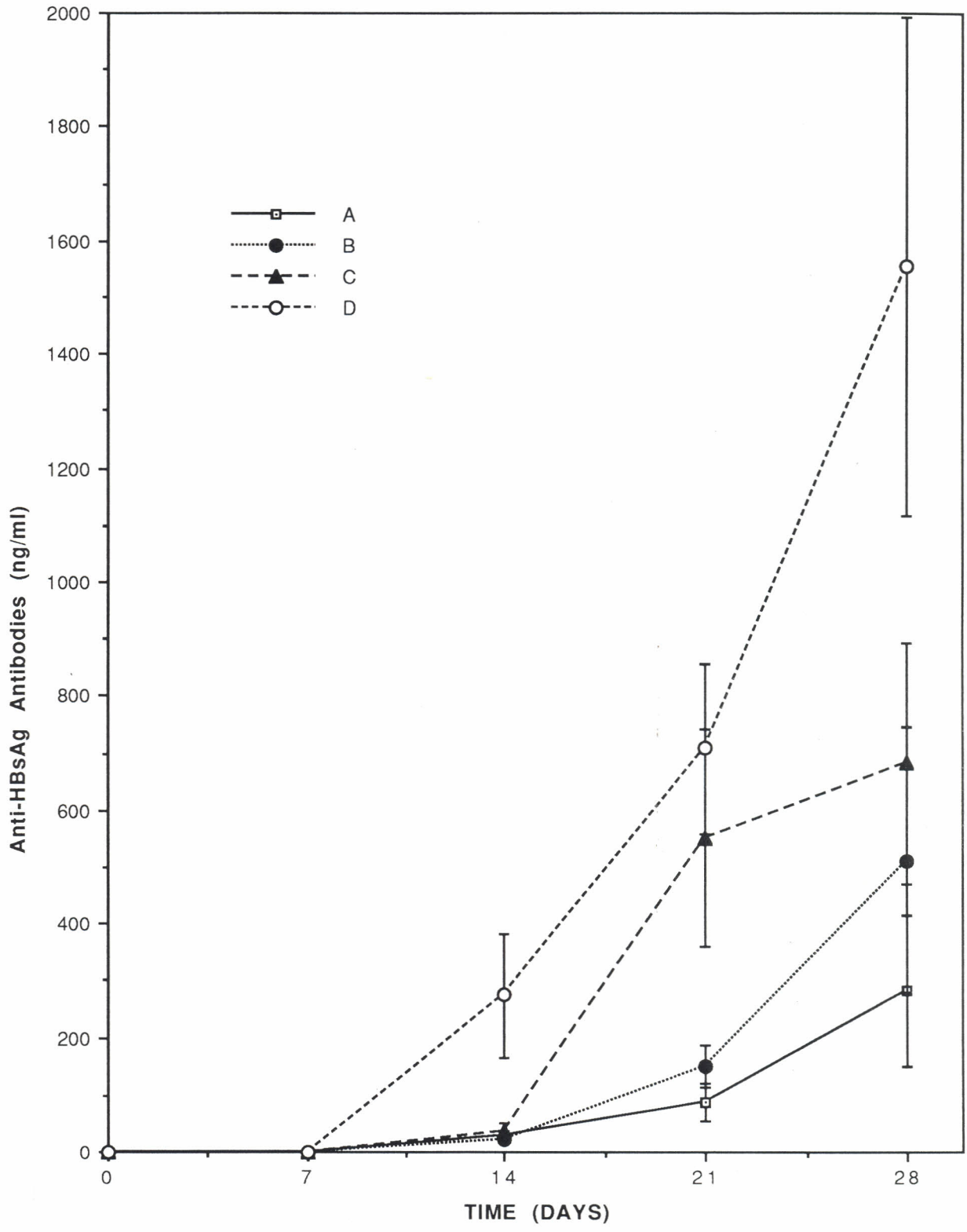



**Figure III: Mean levels of guinea pig anti-HBsAg, $p < 0.05$**

The rate of increase of antibody levels increased with ascorbic acid dosage, i.e the rates for groups A, B, C & D were 9.35, 16.79, 27.37 & 56.13 respectively (see table IV). However there was no statistical significant difference ($p > 0.05$) in the rate of change.

| Group | Rate |
|---|---|
| A | 9.35 |
| B | 16.79 |
| C | 27.37 |
| D | 56.13 |

*Table IV: Rates of change of guinea pig anti-HbsAg antibodies in the different ascorbic acid dosage groups. $p > 0.05$*

# CHAPTER FOUR

## 4.0 DISCUSSION AND CONCLUSION.

### 4.1 Effect of ascorbic acid on immunuglobulin levels.

Two of the experiments investigated the effect of ascorbic acid on antibody levels. The results in section 3.1 showed that the levels of IgG in the three dosage groups B, C and D were significantly higher than those of the control group A ($P < 0.05$). But amongst the dosage groups B, C and D, there was no significant difference in immunoglobulin levels. It was therefore apparent that ascorbic acid may play a role in the synthesis of immunoglobulins. Absence of significant difference amongst the dosage groups could be due to lack of antigenic stimulation as suggested by Prinz *et al.*, (1980). It is possible that ascorbic acid may effect a non-specific net increase in the synthesis of immunoglobulins resulting in raised levels of circulating antibodies. Alternatively, these results are compatible with the suggestion given by Prinz *et al.*, (1980) that ascorbic acid enhances specific antibody synthesis and since in this experiment the animals were not challenged with any antigen this could not be observed.

The experiment where the animals were innoculated with HBsAg support the later hypothesis i.e ascorbic acid enhances the production of antibodies following antigenic stimulation. There was a significant difference in immunoglobulin levels in

all the groups ($P < 0.05$). The antibody levels increased with ascorbic acid dose and with time for example on day 21 the antibody levels were 89.5ng/ml, 150.6ng/ml, 552.04ng/ml and 708ng/ml and on day 28 the levels were 283.4ng/ml, 512.4ng/ml, 682.2ng/ml and 1554.7ng/ml for groups A, B, C and D respectively. This is an indication that asorbic acid enhances immunoglobulin levels on antigenic stimulation. However, from the results, it does not seem to affect the primary response because there was no significant difference ($P > 0.05$) in antibody levels in the early stage of humoral resonse for example the levels on day 7 were 0.84ng/ml, 0.38ng/ml, 0.87ng/ml and 0.42ng/ml for groups A, B, C and D respectively. This is contrary to the findings of Prinz *et al.*, (1980) who observed significantly higher levels of antibodies in the primary response. However they innoculated their animals with Sheep Red Blood Cells (SRBC) which is a T-cell dependent antigen. In their experiment the vitamins effect was only limited to the early phase of primary response so they suggested that ascorbic acid augmments antibody production by acting on B cells via T-lympocytes. But in this case the antigen used was T-cell independent and still there was a significant difference in antibody levels especially after the primary response. This suggests that apart from the effect via T-helper cells, the vitamin could also directly affect the B cells to enhance the production of immunoglobulins against specific antigens. The exact role played by vitamin C

in this process is therefore not yet very clear. Since the significant difference in antibody levels in this experiment was mainly after the primary response, it could be suggested that the vitamin is involved more in the metabolic systems governing the production of antibodies in the cells already in a state of secondary responsiveness than with the response of those cells to primary conditioning by the antigen.

Several experiments have shown that ascorbic acid reduces the severity and duration of the common cold (Briggs 1984; Baird *et al.*, 1979; Pitt & Costrini, 1978; Mink *et al.*, 1987; Elwood *et al.*, 1977) and it has also been shown that during viral infection luecocyte ascorbic acid levels decrease (Fraser *et al.* 1980). From the results of the experiment reported herein, it is possible that one of the roles of ascorbic acid in viral infections is to enhance the humoral response. This would explain why the leucocyte ascorbic acid levels do go down as observed by Fraser *et al.*(1980), which could be due to the utilization of vitamin C in the synthesis of anti-viral immunoglobulins.

## 4.2 Effect of ascorbic acid on T-cell function

The results of this experiment show that ascorbic acid did not affect T-lympocyte proliferation on mitogenic stimulation. Anthony *et al.*, (1980) observed a defect in cell mediated immunity in ascorbic acid deficiency, but no effect of ascorbic acid on T-cell blastogenesis which is in agreement

with the results of this experiment. These results indicate that the defect may not be due to the cells failure to transform but rather a defect in one of the functions of the lymphocytes. Bergesten *et al.*, (1990) suggested that the high concentration of ascorbic acid in the lympocytes may indicate its involvement in the recognition of infectious agents and the subsequent biosynthesis of lymphokines. Since ascorbic acid did not enhance proliferation hence no effect in cell numbers, the defect in ascorbic acid deficiency could reflect a dysfunction of the T-lympocyte rather than reduction in numbers.

## 4.3 Conclusion

On the basis of the results of the experiments above,the following conclusions may be drawn

1. Ascorbic acid is involved in the production of immunoglobulins.

2. Ascorbic acid does not enhance T-Cell proliferation following mitogenic stimulation.

3. Ascorbic acid enhances humoral response against viral antigens.

## 4.4 Suggested further work

From the findings of the experiments above the following areas are recomended for further invsetigation.

1. The role of vitamin C in enhancing the production of immunoglobulin against specific antigens.
2. To find out which phase (primary or secondary) of humoral response is the vitamin involved in.

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# APPENDICES

## I: GUINEA PIG WEIGHTS

Experiment I

### Group A

Week

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 550 | 510 | 545 | 525 | 365 |
| 2 | 577 | 517 | 442 | 366 | D |
| 3 | 245 | 258 | 279 | 250 | 210 |
| 4 | 650 | 585 | 605 | 460 | 430 |
| 5 | 680 | 605 | 593 | 550 | D |
| 6 | 265 | 255 | 297 | 275 | 220 |
| 7 | 525 | 562 | 572 | 558 | D |
| 8 | 280 | 212 | 215 | 255 | 225 |

### Group B

| | | | | | |
|---|---|---|---|---|---|
| 1 | 510 | 516 | 549 | 535 | 555 |
| 2 | 480 | 458 | 485 | 495 | 515 |
| 3 | 575 | 595 | 600 | 630 | 632 |
| 4 | 615 | 615 | 620 | 645 | 655 |
| 5 | 545 | 482 | 545 | 547 | 560 |
| 6 | 435 | 477 | 449 | 445 | 425 |
| 7 | 350 | 370 | 390 | 405 | 450 |
| 8 | 500 | 495 | 530 | 495 | 505 |

### Group C

| | | | | | |
|---|---|---|---|---|---|
| 1 | 580 | 535 | 570 | 597 | 558 |
| 2 | 468 | 467 | 492 | 515 | 545 |
| 3 | 295 | 309 | 324 | 330 | 345 |
| 4 | 625 | 665 | 635 | 615 | 660 |
| 5 | 575 | 570 | 554 | 564 | 600 |
| 6 | 595 | 575 | 578 | 618 | 619 |
| 7 | 280 | 316 | 360 | 400 | 445 |
| 8 | 520 | 510 | 530 | 545 | 495 |

Experiment III

Group A

| | | | | | |
|---|---|---|---|---|---|
| 1 | 440 | 380 | 370 | 326 | D |
| 2 | 563 | 496 | 469 | 426 | 438 |
| 3 | 312 | 304 | 250 | 222 | D |
| 4 | 412 | 402 | 390 | 360 | 370 |
| 5 | 748 | 725 | 580 | 502 | 487 |

Group B

| | | | | | |
|---|---|---|---|---|---|
| 1 | 540 | 426 | 450 | 478 | 480 |
| 2 | 340 | 290 | 310 | 310 | 330 |
| 3 | 330 | 270 | 320 | 310 | 320 |
| 4 | 413 | 340 | 332 | 342 | 361 |
| 5 | 669 | 614 | 460 | 440 | 468 |

Group C

| | | | | | |
|---|---|---|---|---|---|
| 1 | 448 | 398 | 390 | 377 | 444 |
| 2 | 430 | 402 | 397 | 379 | 420 |
| 3 | 595 | 532 | 430 | 400 | 420 |
| 4 | 523 | 430 | 385 | 428 | 452 |
| 5 | 460 | 396 | 383 | 358 | 352 |

Group D

| | | | | | |
|---|---|---|---|---|---|
| 1 | 460 | 397 | 379 | 368 | 350 |
| 2 | 365 | 316 | 360 | 342 | 280 |
| 3 | 369 | 340 | 320 | 322 | 298 |
| 4 | 405 | 328 | 318 | 288 | 300 |
| 5 | 495 | 430 | 420 | 386 | 380 |

## II: COMPOSITION OF BUFFERS AND MEDIA

1. Incomlete RPMI 1640.

| | |
|---|---|
| RPMI 1640 (containing L-glutamine) | 10.4gm |
| $NaHCO_3$ | 1.7gm |
| Antiboitics (Penicillin & Streptomycin) | 10.0ml |
| Distilled water | 1.0l |

2. Complete RPMI 1640

| | |
|---|---|
| Incomplete RPMI 1640 | 90.0ml |
| Feotal Calf Serum | 10.0ml |

3. Carbonate Bicarbonate Buffer (0.05m, pH 9.6)

| | | |
|---|---|---|
| $Na_2CO_3$ | | 1.50g |
| $NaHCO_3$ | | 2.93g |
| $NaN_3$ | 0.20g | |
| Distilled Water | | 1.00l |

4. Phosphate Buffered Saline (PBS pH 7.4)

| | |
|---|---|
| NaCl | 8.0g |
| $KH_2PO_4$ | 0.2g |
| $Na_2HPO.12H_2O$ | 2.9g |
| KCL | 0.2g |
| Make to 1000ml | |

5. PBS-Tween (pH 7.4)

| | |
|---|---|
| PBS | 999.5ml |
| Tween-20 | 0.5ml |

6. PHA Buffer (pH 7.2)

| | | |
|---|---|---|
| $KH_2PO_4$ | | 3.1g |
| $Na_2PHO_4$ | 7.9g | |
| Nacl | | 4.5g |
| $NaN_3$ | | 2.0g |
| Sucrose | | 10.0g |
| Normal Rabbit Serum | | 10.0ml |

Dissolve in one litre of distilled water.

III CONTENTS OF ASCORBIC ACID DEFICIENT DIET

| ITEM | %in | PROTEIN | FAT | FIBRE | ASH | CALCIUM | PHOS | M.E |
|---|---|---|---|---|---|---|---|---|
| Unit | | %/kg | %/kg | %/kg | %/kg | %/kg | %/kg | Kcal |
| Wheat | 0.520 | 6.240 | 1.040 | 1.56 | 2.6 | 0.026 | 0.2132 | 1674 |
| Pollard | 0.320 | 5.376 | 1.344 | 2.624 | 2.624 | 0.0352 | 0.2432 | 931 |
| Yeast | 0.020 | 0.900 | 0.008 | 0.008 | 0.118 | 0.0020 | 0.0280 | 53 |
| Bone | 0.020 | 0.540 | 0.100 | 0.064 | 1.400 | 0.5800 | 0.2800 | 0 |
| Fishmeal | 0.100 | 5.500 | 1.200 | 0.090 | 1.500 | 0.7000 | 0.3500 | 246 |
| PMX Calf | 0.005 | 0.000 | 0.000 | 0.000 | 0.000 | 0.0000 | 0.0000 | 0 |
| PK piglet | 0.005 | 0.000 | 0.000 | 0.000 | 0.000 | 0.0000 | 0.0000 | 0 |
| Salt | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 | 0.0000 | 0.4500 | 0 |
| TOTAL | 1.000 | 18.560 | 3.690 | 4.350 | 8.690 | 1.3400 | 1.1100 | 2905 |

*The constituents of the ascorbic acid deficient diet with their nutritional values.*

KEY

| | |
|---|---|
| %in | Percentage inclusion |
| Ash | Asphalt |
| Phos | Phosphate |
| M.E | Metabolic Energy |

| VITAMINS | VitA | VitE | Thiam | Ribo | Pant | Biotin | Folic | Cholin | B12 | Niacin | vitC |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Unit | I.U | mg | mg | mg | mg | mcg | mcg | mg | mcg | mg | mg |
| Wheat | 0 | 8.06 | 2.70 | 0.57 | 7.02 | 52.0 | 222 | 405 | 0 | 29 | 0 |
| Pollard | 0 | 9.57 | 6.37 | 0.64 | 5.63 | 0.0 | 454 | 298 | 0 | 32 | 0 |
| Yeast | 0 | 0.04 | 1.89 | 0.77 | 2.28 | 100.0 | 180 | 96 | 0 | 10 | 0 |
| Bone | 0 | 0.00 | 0.00 | 0.02 | 0.00 | 1.0 | 7 | 20 | 1 | 0 | 0 |
| Fishmeal | 0 | 0.00 | 0.56 | 0.15 | 0.46 | 4.7 | 140 | 405 | 7 | 4 | 0 |
| PMX Calf | 56000 | 107.50 | 4.00 | 14.00 | 20.00 | 240.0 | 2000 | 0 | 60 | 80 | 0 |
| Pkpiglet | 32000 | 60.00 | 4.00 | 10.00 | 60.00 | 300.0 | 4000 | 800 | 22 | 60 | 0 |
| Salt | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.0 | 0 | 0 | 0 | 0 | 0 |
| *TOTAL* | *88000* | *185.17* | *19.52* | *26.16* | *95.39* | *697.70* | *7003* | *2024* | *90* | *215* | *0.0* |

*The different vitamins and their amounts in the ascorbic acid deficient diet.*

Units in mg/kg dry feed.

KEY

| | |
|---|---|
| %in | Percentage inclusion |
| vit. | Vitamin |
| Thiam. | Thiamine |
| Ribo. | Riboflavin |
| Pant. | Pantothenic Acid |
| Folic | Folic Acid |
| Cholin. | Choline |
| I.U | International Units |

| | A M I N O A C I D S | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | meth | cyst | lys | tryp | threo | isol | hist | val | leuc | erg | phen | gly |
| Wheat | 0.13 | 0.16 | 0.21 | 0.09 | 0.18 | 0.36 | 0.09 | 0.36 | 0.52 | 0.31 | 0.41 | 0 31 |
| Pollard | 0.06 | 0.08 | 0.22 | 0.07 | 0.16 | 0.22 | 0.10 | 0.25 | 0.32 | 0.30 | 0.22 | 0 31 |
| Yeast | 0.02 | 0.01 | 0.07 | 0.02 | 0.05 | 0.04 | 0.03 | 0.05 | 0.06 | 0.04 | 0.04 | 0 03 |
| Bone | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 00 |
| Fishmeal | 0.15 | 0.07 | 0.41 | 0.07 | 0.25 | 0.30 | 0.19 | 0.31 | 0.43 | 0.41 | 0.26 | 0 60 |
| PMX calf | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 00 |
| PK piglet | 0.01 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 00 |
| Salt | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 00 |
| TOTAL | 0.37 | 0.31 | 0.92 | 0.25 | 0.64 | 0.93 | 0.40 | 0.96 | 1.33 | 1.07 | 0.93 | 0 92 |

*The different amino acids and thier quantities in the ascorbic acid deficient diet.*

Units: %/kg dry feed.

KEY

| | | | |
|---|---|---|---|
| Meth. | Methionine | Hist. | Histidine |
| Cyst. | Cystine | Val. | Valine |
| Lys. | Lysine | Leuc. | Leucine |
| Tryp. | Tryptophan | Arg. | Arginine |
| Threo. | Threonine | Phen. | Phenylalanine |
| Isol. | Isoleucine | Glyc. | Glycine |

| MINERALS<br>Units | Sodium<br>% | Potass<br>% | Magnes<br>% | Sul<br>% | Mangan<br>mg/kg | Iron<br>mg/kg | Copper<br>mg/kg | Zinc<br>mg/kg | Selen<br>mg/kg |
|---|---|---|---|---|---|---|---|---|---|
| Wheat | 0.03 | 0.26 | 0.06 | 0.00 | 32.34 | 26.00 | 5.51 | 7.28 | 0.03 |
| Pollard | 0.02 | 0.28 | 0.08 | 0.07 | 36.80 | 32.00 | 3.84 | 33.76 | 0.24 |
| Yeast | 0.00 | 0.03 | 0.00 | 0.00 | 0.11 | 2.00 | 0.66 | 0.77 | 0.03 |
| Bone | 0.00 | 0.65 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Fishmeal | 0.03 | 0.18 | 0.07 | 0.02 | 12.00 | 79.00 | 2.45 | 19.50 | 0.25 |
| PMX Calf | 0.00 | 0.00 | 320.00 | 0.00 | 96.00 | 72.00 | 24.00 | 160.00 | 0.16 |
| PK piglet | 0.00 | 0.00 | 0.00 | 0.00 | 80.00 | 320.00 | 80.00 | 280.00 | 0.20 |
| Salt | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| TOTAL | 0.09 | 1.40 | 320.23 | 0.10 | 257.26 | 531.00 | 116.45 | 501.31 | 0.91 |

*The minerals and their quantities in the ascorbic acid deficient diet.*

*KEY*

| | |
|---|---|
| Potass | Potassium |
| Magnes | Magnesium |
| Sul | Sulfur |
| Mangan | Manganese |
| Selen | Selenium |