GLUCOSE AND ASCORBIC ACID CONTENT OF BLOOD AND TISSUES OF NORMAL AND INSULIN INJECTED RABBITS

by

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INTRODUCTION

The specific work described in this thesis is to be an inquiry into the effect of insulin and/or its induced hypoglycemia on the levels of ascorbic acid in the blood and in various tissues of the rabbit. In addition, morphological changes in the adrenal cortex will be observed. The rabbit is capable of synthesizing ascorbic acid and no question of a primary deficiency is being raised.

Two basic relationships provide a conceivable link between hypoglycemia and adrenal cortical function. Ascorbic acid has been the subject of various researches attempting to implicate it in cortical steroid synthesis, either as a substrate or as a catalytic agent (2), (16), (44), (69), (7), (19), (45), (67). Evidence that glucose is a precursor of ascorbic acid is conclusive (12), (29), (30), (34) and the possibility that chronic or subchronic changes in blood glucose levels might alter synthesis of ascorbic acid will on this basis be partially explored.

It is intended that the subject animals should be exposed to a minimum of extraneous factors which might in themselves cause changes in adrenal cortical function or ascorbic acid levels. By maintaining normal diet and environment at least part of this requirement is met. The only exogenous substance to be used is insulin; a discussion of its possible primary effects will be entered upon later.

PURPOSE

The purpose of this inquiry is to find a possible relation between the frequent hypoglycemic manifestation of abnormal carbohydrate metabolism in bovine ketosis (59), (72) and the pituitary-adrenal cortical damage frequently found in this disease (14), (24), (25), (64).

REVIEW OF LITERATURE

Mechanisms of Adrenal Steroid Production

The stimulus to much of the investigation of steroid synthesis can be credited to Marthe Vogt (73), who established in the course of a number of perfusion experiments on various species that storage of cortical hormone is virtually non-existant. By measuring steroid content of efferent blood of the adrenal gland she found measurable increases after stimulus. When chemical analysis of glands was made at corresponding times almost no hormone could be found. The search for precursors and the train of events leading to the elaboration of the steroid then began, and is still in progress.

With identification of the steroid nature of cortical and gonadal hormones logical precursors were considered and subjected to isotope studies of recovery in the elaborated hormones. Acetate (11), (40), (60) and cholesterol (56), (57) were found to be major steps in the synthetic sequence. As out-

lined by Hayano and his associates (26) on the basis of their own work and that of previous and contemporary investigators, the sequence has been identified as proceeding from cholesterol to pregnenolone to progesterone to deoxycorticosterone, and then to the completed steroid by an 11 beta hydroxylation. The 17 hydro-series of compounds is formed in the 17 hydroxylation of progesterone and then follows the same progression to the secreted state.

Regulation of Adrenal Steroid Production

The regulation of cortical hormone release is accomplished by changing the levels of adrenocorticotrophic hormone (ACTH) released from the anterior pituitary. How the pituitary itself is regulated is subject to some dispute. The classical assumption is that as carbohydrate demands increase sympathetic stimulation and attendant epinephrine release occur to trigger ACTH secretion. This does not account for all of the phenomena associated with the stress syndrome, particularly at a local level. Sayers and Sayers (61) are of the opinion that the available level of cortical steroids at a given moment serves as the regulatory device for ACTH release; as the requirement and use of the steroid increases it is reflected in the concentration of hormone in circulating fluid at the pituitary, which reacts as necessary.

The point of regulation by ACTH is established at some point where the basic synthetic reaction is slowest, rather than at a point where synthesis would proceed in the absence

of hormone. This rate limiting step in synthesis is probably between cholesterol and pregnenelone, an early step in the sequence (27). Sayers and Sayers also support the idea that ACTH works at a point beyond the formation of cholesterol. It has been further determined that the pituitary hormone will not accelerate incorporation of labeled acetate into cholesterol (70). It is interesting that Hechter, et. al. (27) are of the opinion that on the basis of their work ACTH calls forth only about one percent of the cortical potential in terms of steroid hormones.

Ascorbic Acid in Adrenal Steroid Synthesis

Cholesterol and ascorbic acid have been subject to association due to the relatively high levels of both substances found in the adrenal. It has been found for example that the drop in cholesterol and depletion of measurable ascorbic acid proceed at about the same rate when the gland is stimulated (63), (69). When the subject is made deficient in ascorbic acid however, it is found that loss of cholesterol will proceed much more slowly than loss of vitamins due to deprivation (69). The question of continued synthesis of cholesterol with all required precursors present compared against that of ascorbic acid with none, seems not to have been considered. In another study of similar conditions it was found that adrenal (and other tissue) cholesterol was not

changed in either direction during the deficiency state. Booker and his co-workers observed a drop in blood ascorbic acid following chronic administration of high levels of cholesterol, and found that ascorbic acid administration would cause cholesterol increases (9). Circumstances of dosage would suggest that some other nonspecific mechanism may be functional and invalidate the work.

In the progress or as the object of various studies of scorbutic animals changes in adrenal weight have been measured and have been consistently found increased (6), (7), (19), (31), (44). An obvious question arises --- whether the enlargement was due to cortical activity incited by the stresses of inanition, or resultant from a deficiency of ascorbic acid and the hypertrophy undertaken to provide sufficient hormone by an inefficient synthetic mechanism.

When the normal adrenal cortex is stimulated to secrete its hormones for regulation of carbohydrate metabolism, there is a decrease in the amount of ascorbic acid as such in the gland (24), (32), (54), (69), (76). Rinfret (54) injected extracts of infant pituitary into hypophysectomized rats and noted a fall in adrenal ascorbic acid. Wexler, et. al. (76) provided a non-specific stimulus by injecting a bacterial polysaccharide commercially prepared as a pyretic, and noted a fall in adrenal ascorbic acid. The ascorbic acid drop was used as an index of cortical stimulation from the pituitary. That this is valid procedure is confirmed in comparative

studies of ascorbic acid loss in stressed normal, and ACTH treated hypophysectomized rats (63). No differences were detected. Slusher and Roberts (67) stressed rats with laparotomy and found a release of ascorbic acid which reached its peak in about 15 minutes. Maximum corticoid production occurred up to 15 minutes following this peak. The technique used in this experiment measured the vitamin in the effluent blood of the adrenal. Vogt (74) was unable to demonstrate this in her pioneering attempts, probably due to factors of time. The method of determination was the same.

Deane and Morse (16) found that ascorbic acid was retained in the adrenal cortices of rats submitted to starvation, and noted that the lipids presumed to be precursors of the steroids had disappeared. In hypophysectomized rats they did find a drop in ascorbic acid proportional to the ability of the cortex to produce its hormones, but on the basis of the starvation experiments assumed that the vitamin has no function in steroid elaboration. Other workers have produced evidence that the vitamin is not required in production of steroids. Scorbutic animals stimulated by ACTH produced greater amounts of steroid than non-stimulated deficient animals, showing that cortical function exists in an avitaminosis C (19), (31), (45). In fact the steroid output of ascorbic acid deficient guinea pigs in certain experiments has been shown to be greater than their normal control animals (45), (49).

Eisenstein and Shank (19) found a variance in adrenal weight which corresponded inversely with the intake of asdorbic acid. They also evaluated cortical function in deficient guinea pigs by observing the decreases in eosinophiles, the greater decrease following administration of ACTH. ACTH also increased survival time and delayed scorbutic symptoms. In another similar experiment (31) the adrenal weights as related to body weight showed a three fold increase in untreated scorbutic animals. Deficient animals treated with cortisone had adrenals about one-half normal size and the scorbutic subjects given ACTH developed glands about five times normal weight. In addition there was demonstrated a great increase in secreting granules in the ACTH treated glands, which indicated that although the gland was undergoing a hypertrophy in response to lack of an important metabolite, it would function in spite of this lack.

Some of the experiments above could be used to defend the proposal that ascorbic acid exerts a restraining influence on cortical activity. Experiments with insulin sensitivity show that the hypoglycemia of insulin is potentiated by administration of ascorbic acid (2). The same paper notes that the vitamin does not cause changes in insulin levels or glycemia in normal animals. Failure of this potentiation is demonstrated with adrenalectomized rats treated with insulin. No difference can be seen in the response of ascorbic acid treated and non-treated groups (3). Dury and Dury (18)

contradict certain parts of this work in an experiment in which normal rats did not develop insulin hypoglycemia when protected by ascorbic acid. The difference in response to similar situations is not readily explainable. In the former work, the ascorbic acid was given after insulin, and in the latter investigation the animals were pretreated. In both cases the levels of vitamin C administered were sufficient to cause blood levels many times normal.

Evidence in favor of a positive role of ascorbic acid in steroid synthesis has been studied with isotope techniques. Use of cholesterol $4O^{14}$ caused formation of several of the intermediates described in the review by Hayano and his group (26) mentioned previously. When ascorbic acid was added to the system genesis of adrenal steroids was accelerated, (57). Bacchus (4) also describes an enhanced steroid synthesis when ascorbic acid is added, and in addition states that 11 Beta hydroxylation of deoxycorticosterol decreased in ascorbic acid deficiency. The significance of this particular step in the synthetic process will be clarified in the review of Hayano's work.

One group of investigators has demonstrated an <u>in vitro</u> acceleration of steroid synthesis by ascorbic acid (37). The system used was a mitchondrial suspension with ATP and either DPN or nicotinamide at pH 6.6, in which deoxycorticosterone was hydroxylated at the 11 Beta carbon to give 10 percent yield of corticosterone in thirty minutes. Addition of ascorbic acid

raised the output to 20 percent in 10 minutes.

As well as attacking the problem of mapping the reactions concerned in steroid synthesis, Hayano's associates (35), (26) have attempted to localize the activity of ascorbic acid in cortical chemistry and conclude that it serves as an inhibitor of the 11 Beta hydroxylation reaction, between deoxycorticosterone and corticosterone. It is interesting to note that this reaction is specific to the adrenal gland (27) and that the adrenal contains by far the greatest concentration of ascorbic acid on a weight basis of all the body organs (62). If it is correct to assume an inhibitory function for the vitamin the presence or absence of ascorbic acid at a given time might influence release of the steroid when the influence of ACTH is absent or negligible, as in a non-emergency situation. In fact, if as postulated, ACTH secretion is a function of steroid levels in blood, then the contant "leakage" which presumably takes place in any unstimulated uncatalyzed reaction would continue, and a level of steroid high enough to maintain integrity of the animal and high enough to block the pituitary would occur. It has been demonstrated that ascorbic acid disappears from the cortex prior to the peak of steroid production (67). If ascorbic acid was an inhibitor of the leakage inherent in the system, it would have to be removed or overwhelmed at the time ACTH reaches the cortex. The necessity of preventing even slight loss indiscriminately into the circulation is obvious when

considering the work of Vogt (73) in which it was found by measurements of arterial steroid levels that the hormone disappears very quickly in the tissues. The results of such loss in the scorbutic animal are not known, but may if they exist serve to delay the ravages of the deficiency. Increased cortical activity is known to accomplish this (19), (31) and the increased steroid output in scorbutic animals, presumably of ACTH origin has already been described (45).

As well as the dispute over the effect of ascorbic acid on steroid production, questions of its nature of action are not solved. Most consideration of this problem assumes some exercize of the great oxidation-reduction activity of ascorbic acid (38). If this reaction is the base of its function, the vitamin would be converted to dehydroascorbic acid and would presumably remain at the site of conversion to be reduced and returned to use. The equilibrium is strongly in favor of ascorbic acid (10). Histochemical (5) and isotope (58) studies have shown that under non-emergency conditions there is almost no dehydroascorbic acid in the cortex at a given time, and the isotope studies have shown in addition that only minute quantities of the ascorbic acid is irreversibly oxidized to diketogulonic acid, the principle metabolic end product, or other metabolites. The mechanism for reconstitution is probably a sulfhydryl containing system, perhaps glutathione. The ready reversibility of this reaction can be presumed to indicate that the reactant is relatively

immobile, and is used over and over. The results of Vogt's perfusion experiments in which no efferent ascorbic acid was found after stimulation supported this idea (74), but the later work of Slusher and Roberts (67) has shown a marked output of ascorbic acid by the adrenal and other tissues following adrenal stimulation.

Ascorbic Acid Synthesis

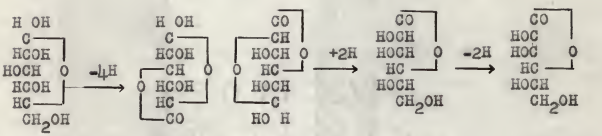
The formation of L-ascorbic acid from D-glucose has been described in theory and experimentally. In addition the entire carbon chain of glucose has been demonstrated to survive the transformation intact.

The basic work relating ascorbic acid to a hexose precursor was done by Jackel and his associates. The technique was to inject labeled glucose, and stimulate synthesis of ascorbic acid by the use of the depressant, chloretone. They found a significant amount of label converted in a 24 hour period, about 0.3 percent. This figure corresponds to normal conversion in the untreated animal (34). Horowitz and his collaborators recovered L-6-C^{14} ascorbic acid after feeding D 1 C^{14} glucose to chloretonized rats (26), and in the converse of this experiment, recovered ascorbic acid labeled at the terminal carbon from glucose labeled at C 1 (29). From these studies it was clear that the molecule of glucose survived the synthetic process.

The next problem to be attacked was that of the synthetic

sequence --- the compounds intermediate between glucose and ascorbic acid. One of the first to be identified was glucuronic acid. Albino rats administered this substance were shown to produce a greater amount of ascorbic acid than untreated controls (30). It is possible that this observation explains the increased ascorbic acid synthesis following the administration of depressants, particularly barbiturates and chloretone. These and many other sedative or anesthetic drugs cause the production of glucuronic acid from glycogen during the detoxication process in the liver, and when low levels are used may stimulate enough glucuronate formation to increase ascorbic acid formation.

By using certain theoretical intermediates Isherwood, et. al. (32), (33), have demonstrated that several are capable of production of ascorbic acid. From this work, a suggested sequence is as follows:



None of the literature reviewed has considered directly the possibility of attaining a level of glucose low enough

to impede ascorbic acid synthesis. Either of two considerations seems logical in this problem. The stated normal conversion of glucose to ascorbic acid daily is on the order of 0.3 percent. It seems possible that a reaction requiring relatively small amounts of substrate would be satisfied in preference to the more massive matabolic reactions, and even at the lethal levels of 10 to 40 mg. percent conversion would take place. On the other hand, if ascorbic acid formation is in a direct equilibrium with metabolic demands and therefore sugar levels, a prolonged decrease of sugar to shock levels could reduce vitamin synthesis to a degree impossible for the subject to tolerate.

The site of formation of ascorbic acid is not established. Several organs, especially the adrenal cortex, the small intestine and liver have shown to contain high levels of the substance. Whether this is a consequence of synthetic reactions at the site or a highly mobile pool of ascorbic acid coupled with local storage mechanisms is not known. The relatively high mass of the intestine and liver would favor the concept of a mobile available supply of the vitamin. Reid has compiled a table showing values for various organs as established by a variety of analytic techniques (62).

Evidence in favor of a synthetic role of the adrenal cortex has been presented in the form of drastically reduced ascorbic acid excretion in adrenalectomized rats (17).

The Effect of Insulin on Ascorbic Acid Synthesis

There are infrequent references in the literature to the effect of administered insulin on blood or tissue ascorbic acid. In each instance levels were lowered. Experimental hypoglycemia induced by insulin in 20 human subjects caused a decrease in blood ascorbic acid in 17 individuals. Blood levels were increased in the other three cases (13). Pancorvo (48), in work on diabetic patients, recorded an average drop in blood ascorbic acid from a normal of 0.8 mg./ 100 ml. to 0.74 mg./ 100 ml. Insulin further reduced these averages to 0.5 mg./ 100 ml., and his data showed that recovery to preinsulin diabetic levels was very slow. He suggested that insulin might do one of three things: assist the oxidation of ascorbic acid, block resynthesis or increase requirements.

Karg (36) devised a procedure for the independent estimation of ascorbic acid and epinephrine in one operation, and then studied the concentration of these substances in the adrenal cortex after insulin administration. He found a decrease in ascorbic acid proportional to the dose of insulin, and found also that by maintaining blood glucose with infusions the drop could be inhibited. The experiment is apparently with a single dose of insulin and seems to indicate that synthesis does depend on substrate levels, and is a highly labile process. The results of depressant administration might be

interpreted the same way, an increase in glucuronic acid leading to increased synthesis. In Karg's experiment there is also a possibility that with diminished levels of blood glucose the adrenal cortex is being stimulated to maintain normoglycemia, with the resultant expenditure or dissappearance (45), (54), (67), (69) of the vitamin.

None of these experiments was concerned with effects of prolonged administration of insulin.

Hypoglycemia

The available literature on hypoglycemia is divided among four groups of reports. Two of these are clinical; two are derived from investigational activity.

Of the clinical hypoglycemias the most common are the endogenous hyperinsulinisms, usually the result of neoplastic activity of the pancreatic islets. A relatively limited amount describes the so-called idiopathic hypoglycemias, some of which have a possible familial basis, others of which may be related to neural disorders. Many of the examples of these types are available only as isolated case reports and are investigated from a clinical rather than physiological viewpoint.

The third group is that in which the effects of exogenous insulin is studied in both normal and diabetic humans and

animals. The fourth contains the results of a variety of experiments which by design or accident resulted in the attainment of a hypoglycemic state.

For the ideal study of hypoglycemic effects on certain processes of metabolism subjects of the idiopathic type might seem more satisfactory than those in which the condition is instituted by insulin administration. However, the condition is noticed only in the human except in rare and almost accidental cases, and immediate efforts are made to begin effective treatment empirically to assure a minimum of damage to the patient. Adrenal cortical hormones are often used for this purpose. No such cases could be used to study other biochemical events.

The most striking observation to be made from these studies is the great difference in dependence on glucose between the central nervous system and the peripheral tissues. Blood levels in idiopathis and insulinoma cases descend to levels at which central nervous metabolism cannot be maintained (42), (66), (20), (18), and the outcome is the typical hypoglycemic convulsion. Levels as low as nine mg./ 100 ml. have been recorded in man (42) and while somatic integrity does not seem to suffer the central nervous system is unable to function and suffers permanent damage. Because the situation usually arises for the first time in infants the convulsive symptoms may not be recognized as specific, and the lack of carbohydrate may go unattended while the patient is sedated in non-

specific convulsion therapy. Certain victims of this syndrome are very sensitive to amino acid or protein intake and a crisis may be precipitated by these substances orally or parenterally (15). Adreno-cortical hormones usually will raise sugar levels to 50 - 90 mg./ 100 ml. even when no adrenal deficiency can be detected (42).

Certain patients have been described in which the hypoglycemia is suspected of being caused by a neural condition (20). Individuals are described who suffer from anxiety or other nervous state and who exhibit apparent secondary hypoglycemia. The involvement of a pituitary-adrenal condition is not described.

Attempts have been made with non-physiological substances to institute hypoglycemia in various species without causing interference or damage to the rest of the body economy by the hypoglycemic agent. In every case concessions have had to be made to side effects, so that few of these agents are satisfactory.

In studies of the etiology of ketosis Richards and Weaver (53) used Synthalin A to lower blood sugar levels, and found that it caused extreme liver damage at levels only slightly above those required to lower blood sugar. The maximum dose was found to be less for mature animals than for calves. Levels were obtained which went below those found in ketosis, but fatalities in older cattle result from the many other effects of the drug. It has been tried in chickens and the rabbit, and following a characteristic initial hyperglycemia,

will produce hypoglycemic convulsions in about 18 hours (8). The use of cobalt chloride ($CoCl_2$) has been explored as an agent for the destruction of the alpha cells of the pancreas and their supposed hyperglycemic factor. The hypoglycemia is undependable and compounded with other toxic effects. The net result is often a rise in blood sugar (75). Mirsky has reported an observed hypoglycemic effect of certain auxins or plant hormones but no steps toward a clinical application have been taken (43). Environmental changes have been used to change blood sugar levels, the most striking being the effect of increased temperature. At 120 degrees Fahrenheit and in relatively dry surrounding atmosphere glucose dropped as much as 22 percent, with only a six percent dehydration, over a period of four hours. The change was apparently due to increased metabolism, because no spillage was detected in the urine (35).

Activity of Insulin

None of the drugs or procedures is effective or as easily controlled as insulin in producing a hypoglycemic state. Insulin and its function as a physiological hypoglycemic agent are well described in standard texts (23). The most common view of its activity is as an agent enhancing membrane permeability. Subjects deprived of endogenous insulin metabolize glucose as effectively as intact animals if levels of blood

sugar are maintained at a sufficiently high level --- about four times normal (41). The effect in such a case would be that of raising a concentration gradient which would force glucose across the cell membrane to be phosphorylated. Park, Bornstein and Post have been able to localize the effect of insulin at a point preceding phosphorylation by using in vitro preparations of rat diaphragm and comparing amounts of free and phosphorylated glucose before and after insulin. This work also suggests an effect in the mechanism of crossing of the cell barrier (51). The classical descriptions of insulin include the demonstration that a homogenate of metabolizing cells with no intact cellular barriers is capable of metabolism at normal glucose concentrations without addition of insulin. Park and Johnson (52) have shown localization of free muscle glucose in extracellar fluids without insulin and in intracellular fluids with insulin. They also noted that galactose, and therefore presumably glucose, could be found in equal concentrations on either side of the membrane of central nervous system cells without insulin. Their conclusion from this information was that insulin is not needed in glucose transport in the brain and cord. The failure of entry of galactose into nervous energy metabolism is apparently disregarded here.

It should be theoretically possible to separate the effects of insulin and its hypoglycemia by maintaining the glycemic level of insulinized animals artificially. The

literature is almost barren on this subject, the few references being incidental to other work. An example is Karg's work (36) in which he gave insulin and sugar together and found an inhibition of ascorbic acid loss from the adrenal. It has been found in this laboratory that regulation with any constancy is very difficult.

The effects of a hypoglycemia should be more easily observed in a subject maintained for extended periods in a state of low blood sugar, and again the literature lacks in discussion of such procedure. Only one group of researchers was found who had successfully extended hypoglycemia for purposes of study. Goats were treated with insulin and held in the vicinity of 30 mg. glucose percent for one week (65). As will be described later, a reasonably well controlled technique for maintenance of this condition in rabbits over periods as long as thirty days has been evolved for the purposes of this experiment.

There is a possible theoretical complication to the use of insulin in an experiment intended to investigate change in ascorbic acid levels resulting from changes in blood sugar. Insulin has been demonstrated to have an effect on synthesis of fatty acids from glucose via routes other than the glycolytic scheme (1), (21). Whether this is due to a specific effect on one of the reactions in the alternate pathway, or simply the mass action effect of glucose forced into the cell in excess of glycolytic capacity is not known. The route of this synthesis

has been found by Felts, Duell and Chaikoff to go through the phosphogluconate pathway from glucose-6-phosphate (21). The synthesis of ascorbic acid in biological systems may conceivably follow a part of this route, and if insulin acts on one of these reactions it could directly cause an increased synthesis independent of sugar levels.

MATERIALS AND METHODS

Organization

The subject animals were mature rabbits of both sexes. Within each group the experimental and control animals were of the same age, and as nearly as possible of the same strain. They were purchased from the rabbitry of Lottie Greer of Manhattan, Kansas.

Organization of the experimental groups was as follows:

Group	Number of Rabbits	Treatment	Duration
I a	6	U-40 insulin, TID	32 days
I b	3	blank injection, TID	32 days
I c	3	starvation	16 days
II a	6	U-40 insulin, TID	18 days
II b	6	blank injection, TID	18 days
III a	5	U-40, 50 IU, one dose	7 hours
III b	4	blank injection	7 hours
IV a	3	glucose 10%, 50 ml/hr	3 hours
IV b	2	PSS 50 ml/hr	3 hours

Each major group was obtained separately, and was randomized into subgroups by numbering the subject animals and having a disinterested individual separate similarly numbered cards into two groups. Groups III and IV were of the same age and were separated into three sub-groups by the same procedure. The subgroups IVa and IVb were separated arbitrarily because each rabbit constituted an essentially separate experiment. Group IIIb served as control for group III and as negative control for group IV.

Group I served primarily as an exploratory experiment in which the practicability of long term insulin administration to produce hypoglycemia could be determined. The objective established was to carry the rabbits for at least thirty days in a hypoglycemic state, at which time the survivors would be sacrificed. This group provided no experimental data other than the desired information for conduct of succeeding work, and is therefore described in a separate section in the discussion of this paper.

In addition three rabbits were fasted for 16 days, receiving only water as desired. The purpose was to evaluate starvation as a hypoglycemic agency. At the end of this period two animals were administered insulin to cause hypoglycemic shock, and the third was euthanized with sodium pentobarbital. Tissues from each were studied. These results are included in this thesis only as an incidental in the discussion.

Housing of the rabbits was in standard cages of floor

dimensions 14 by 22 inches by 10 inches in height, with half inch wire mesh floor.

Diet throughout the experiment was Purina Rabbit Chow. No additional salt or other dietary substance was added to the intake. Feed was replenished as necessary three times daily, to accomodate the increased intake by insulinized animals.

Experimental Procedure

Group I - Exploration of Methods for Producing Chronic Hypoglycemia. Prior to treatment, blood samples were drawn for determination of normal blood glucose levels of both treated and control groups.

Group Ia was subjected to progressively increased doses of protamine zinc insulin (U-40, Eli Lilly and Company) administered three times daily. The animals were treated in this manner for 32 days, or to the initiation of insulin shock. Daily dosage at this time ranged from 27 - 30 International Units. Blood samples were drawn at approximately 48 hour intervals, and the dosage of insulin in each rabbit adjusted accordingly to hold sugar levels as low as possible without incurring a hypoglycemic reaction.

At the end of 32 days the surviving treated and control subjects were sacrificed and autopsied, and tissues removed for histochemical estimation of ascorbic acid. No chemical analyses were made.

Group II - Changes in Ascorbic Acid during Chronic Insulinism and Hypoglycemia.

Conduct of the experiment. Treatment was begun as in group Ia, with the difference that dosage was determined by the average sugar level and held equal for all rabbits in the group until the last three days of administration. Blood sugar determinations were made at 48 hour intervals initially and more frequently when dictated by hypoglycemia. This program was continued for 18 days after sustained hypoglycemia was produced. Control animals were handled and received blank injections on the same schedule as the experimental group, but blood glucose was not routinely determined. At the end of the 18 day period, insulin dosages averaged 54 I. U. per day per animal.

When collapse of most of the treated animals seemed imminent the group and its control animals were sacrificed over a two day period. Prior to euthanasia blood was drawn for glucose and ascorbic acid determination. Each animal was then administered sodium pentobarbital and autopsied. This method of sacrifice was regarded as least likely to produce changes in ascorbic acid which might accompany the possible corticoid release attendant on the more violent stunning or severance of the jugular. The effect of barbiturate depression on ascorbic acid synthesis was considered to be of no concern because of the very short period of drug influence.

Preparation of Biological Materials for Analysis. Two milliliters of blood for determination of circulating ascorbic

acid were placed slowly into six milliliters of six percent trichloroacetic acid to precipitate the protein and to provide a suitable medium for the reaction, and this preparation was then frozen for later assay of ascorbic acid content. The left adrenal and sections of jejunum and liver were cleaned of surrounding tissue, excessive blood and ingesta; then they were weighed and similarly frozen whole in 7.5 milliliters of four percent trichloroacetic acid for future analysis.

The right adrenal and sections of liver and jejunum were prepared for histochemical evaluation of tissue ascorbic acid. Slices of these same tissues were also stained with hematoxylin and eosin for histologic examination.

Group III - Changes in Ascorbic Acid during Acute Insulinism and Hypoglycemia. Each subject was administered a heavy dose (50 I. U.) of protamine zinc insulin, and arbitrarily sacrificed seven hours later. Control animals were handled and administered blank injections on the same schedule. Preinjection and premortem blood samples and post mortem samples of tissues were obtained and processed for analysis as for group II.

Group IV - Changes in Ascorbic Acid during Hyperglycemia. The rabbits of this group were made hyperglycemic by injection of 150 milliliters of 10 percent glucose in .85 percent NaCl. This solution was given in 50 milliliter doses hourly at multiple injection sites. No spreading factor was used. The subjects were euthanized thirty minutes after the third dose.

The positive control, group IVb, consisted of two rabbits administered .85 percent NaCl in 50 milliliter doses to a total of 150 milliliters.

Experimental Techniques

Drawing and Processing of Blood. The necessity for frequent drawing of blood samples without causing trauma of any kind to the subject was recognized at the outset. The ear veins are most usually used for venipuncture but are very fragile and difficulty in obtaining more than one or two milliliters of blood is common. The only other accessible source of blood is the heart. Heart puncture was ruled out because of occasional mortality, and the stimulation and cortical discharge which would result from handling. Because of this prohibition the following procedure for drawing blood from the ear veins was devised.

Preparation of Subject. An apparatus for restraint of the rabbit was devised so that the head was firmly but comfortably held immobile and the body was in a reasonably comfortable position. Each animal was handled and restrained in this device a number of times prior to the first experimental procedure so that changes due to the apprehension of handling would be uniform.

Both ears were clipped closely at the beginning and whenever hair growth interfered with vision of the ear vessels. When preparing to draw blood the area of puncture was sponged

with xylene. When engorgement developed, the xylene was removed with alcohol which was in turn washed off with water. The ear was then dried with facial tissue. No occlusion of vessels was needed if engorgement was complete.

It was important that each needle be sharpened under magnification frequently, as any irregularities would damage veins to such an extent that further use was difficult. It was important that aspiration be gentle in order that the vessel wall or the numerous valves in the area were not pulled into the needle. This accident would cause severe pain and interfere with restraint. When large quantities of blood were required, the needle was inserted against the direction of flow or even into the artery, but damage to the vessel seemed slightly greater. It was possible to draw blood at hourly intervals over an extended period if vessels were judiciously selected and if all precautions were observed.

Equipment for Drawing and Measuring Blood Samples. For the purpose of the experiment it was necessary to draw four milliliters of blood. A five milliliter syringe was used, rinsed with and containing 0.2 milliliters of 0.1 percent heparin solution. A sterile needle and fresh syringe was used for each drawing.

Twelve five milliliter Luer-Lok syringes of the same lot manufactured by Becton-Dickinson and Company were reserved for the specific work of obtaining and measuring blood samples. Each of these syringes was calibrated by direct fluid connection

with five milliliter by 0.1 milliliter Pyrex Red Line pipettes conventionally used in the measurement of blood aliquots. It was found that the maximum error for five milliliters, the capacity of the syringes, was of the order of ± three percent. The maximum error per milliliter was ± two percent when compared with a two milliliter by 0.01 milliliter Exax pipette. In addition random comparisons of various fractions of a milliliter in tenths were made with similar results. As a result it was decided to discard a procedure of recording the serial of each syringe and a corresponding correction which would apply to the quantitative analysis of contained blood.

Determination of Blood Glucose. The method of Nelson (46) was applied to measurement of blood glucose with minor changes in the procedure for deproteinization. Barium hydroxide specified at 0.3 N and zinc sulfate specified at five percent were used in concentrations of 0.15 N and 2.5 percent respectively. In addition blood was diluted to one milliliter in two milliliters of water rather than one milliliter in 15 milliliters of water. The purpose of these changes was to allow use of the prepared material for estimation of ketones if desired. Blood so deproteinated contains negligible amounts of reducing substances other than glucose. When treated with a prepared copper reagent in alkaline medium, a complex precipitate is formed. Treatment with an arsenomolybdate reagent develops a blue color whose intensity varies with glucose concentration. Color reactions were analyzed

with the Coleman 14 spectrophotometer at 520 millimicrons.

Determination of Blood and Tissue Ascorbic Acid. Blood ascorbic acid was determined according to the method of Roe and Kuether (55). This procedure oxidizes ascorbic acid to dehydroascorbic acid, then converts the entire dehydroascorbic acid content of the sample to an osazone. This product is dissolved in sulfuric acid and the concentration of the orange solution so formed is determined spectrophotometrically.

Tissue ascorbic acid measurement was carried out with this basic technique with modifications suggested by Slover and Newcomer (47). These consisted of altering the concentration of trichloroacetic acid from six percent to four percent, and using 7.5 milliliters of this dilution rather than six milliliters so that no correction need be made for differences between blood and tissue volume.

The use of 0.2 milliliters of 0.1 percent heparin necessitated a correction of plus five percent in blood glucose and ascorbic acid which was included at the time of measurement for convenience.

The values found for tissues were read in terms of total ascorbic acid per sample and were calculated to terms of milligrams ascorbic acid per 100 grams tissue weight:

$$\left(\frac{100 \text{ gm}}{\text{organ or tissue weight}}\right) (\text{mg total ascorbic acid}) = \text{mg ascorbic acid per 100 gm tissue weight}$$

Preparation of Tissues and Blood for Analysis of Ascorbic Acid Content. The chemical determination and histochemical demonstration of ascorbic acid each demand immediate processing of tissues. Because of this conflict an order of precedence was established so that no damaging delays interfered with the effectiveness of either procedure.

In the uniform order of handling which was followed, tissues for chemical analysis were prepared first because of the more pressing need for immediate processing and then appropriate tissues were fixed for histological examination. Deane and Morse (16) have stated that intracellular distribution of the vitamin is altered if tissues are not fixed within three minutes. This has been found true in this laboratory but the distribution in terms of tissue areas did not suffer appreciably through an additional three minutes. It is questionable whether this can be prevented in fixing a whole gland and for the purposes of this work it was assumed sufficient to fix tissues for histochemistry within five to seven minutes of euthanasia. A greater delay would result in solublization of ascorbic acid and migration to intercellular spaces and to the periphery of the organ.

The left adrenal was removed immediately upon death and trimmed of fascia; and sections of jejunum and liver were removed. Each was cleaned of blood or ingesta, blotted and weighed on previously tared cover glasses. Each tissue was then placed in 7.5 milliliters four percent trichloroacetic

acid and frozen at minus 27 - 30 degrees Centigrade for later analysis by the method of Roe and Kuether (55). Blood samples were also preserved in this manner, in a dilution of two milliliters of blood dropped slowly into six milliliters of six percent trichloroacetic acid.

The effects of freezing on storage of ascorbic acid has been investigated in connection with problems of frozen food handling. In a summary of findings related to these problems (50), it is noted that little loss occurs during extensive periods of storage at minus 20 degrees Centigrade and below. The Department of Foods and Nutrition at Kansas State University has also investigated these processes; a constant percentage loss has been noted in freezing and thawing but very slight decrement of vitamin content occurs over long periods of storage (71).

It appears that much of the vitamin C loss in food processing is by leaching in preparation and by enzyme systems not inactivated immediately. In the preparation of samples for these experiments, the former was prevented because no fluid was lost. Enzyme systems were not expected to survive the immediate immersion in the acid deproteinating solution or the freezing process.

From this it would appear that no loss could have occurred from the samples as prepared. However, known aqueous samples were analyzed before and after freezing and an average loss of 26 percent was found, values deviating ± three percent.

Unknown samples of blood were similarly handled and a loss of 23 percent noted. It was decided to use a standard figure of 25 percent to represent loss in freezing.

Frozen tissues and blood were thawed in cold tap water, homogenized and agitated in the storage tube with 0.3 grams acid washed Norit. The time elapsed between removal of samples from low temperature and beginning of the analytic reaction was kept constant from sample to sample. Four milliliters of filtrate were aspirated from the tube using a filtering pipette devised for the purpose. This was necessary because the recommended gravity filtering allowed too great a time lapse, and it was found that centrifugation did not drive colloidal carbon out of the filtrate.

The filtering pipette was made by wrapping the tip of a five milliliter by 0.1 milliliter Pyrex red line pipette with one-half disc of six inch SS analytical filter paper, fastening with elastic, and folding and crimping the end of the resulting cylinder. To drain the pipette, the wet paper was torn off above the tip. Care must be taken that filtered particles do not drop into the receptacle from the paper tip.

Histochemical Demonstration of Ascorbic Acid. For study of area distribution of ascorbic acid the right adrenal and sections of liver and intestine were treated in an alcoholic silver nitrate solution. This was followed by a fixative solution of sodium thiosulfate and sodium nitrate according to the method of Bourne as modified by Lillie (39). This

technique is reputedly specific for ascorbic acid as the only substance of sufficient reducing potential to convert silver nitrate into metallic silver. Sections of this preparation were cut at six microns thickness and mounted without staining. Duplicate sections were stained with hematoxylin and eosin for histologic examination.

EXPERIMENTAL RESULTS

Exploration of Methods for Producing Chronic Hypoglycemia

Prolonged Insulin Administration. The treated animals in this study were administered protamine zinc insulin at the discretionary limits suggested by individual blood glucose assays. This was in contrast to a later group in which the same dosage was maintained in all treated animals according to average blood sugar levels. The quoted blood glucose level at which rabbits display hypoglycemic symptoms is approximately 38 to 45 milligrams per 100 milliliters blood (22), (68). An attempt was made to approach and remain near this low level for a protracted period of time. Because of the peculiar resilience of the subjects and occasional conservatism in the administration of insulin, these levels sometimes rose above a useful range. Each time this occurred, the glucose levels were found to be successively more difficult to reduce.

The dosage was begun at two International Units daily (one sensitive animal went immediately into shock at this

dosage) and at the end of 32 days surviving animals were receiving from 27 to 30 units daily. The glucose levels at incidence of hypoglycemic episodes varied from 12 to 50 milligrams per 100 milliliters. Individual resistance to hypoglycemia and time of survival varied as well.

This group of animals was used during the warmer part of the summer. Food intake of the treated animals was two to three times that of control subjects; this excessive intake of food continued on humid days when control animals refused to eat. The impression in observing these animals was that insulin was actually driving the carbohydrate metabolism at excessive rates, necessitating the high intake of an energy source.

Each of these animals was allowed to develop the shock syndrome, a blood sample was drawn, and euthanasia was performed immediately prior to the expected time of death.

Post-mortem examination of these animals showed hemorrhagic foci at the patellar joints in many cases and subperitoneally in the lumbar region. Other joint areas were irregularly affected. Examination showed that these foci were of mechanical origin in the violent muscular activity of the convulsions. One uniform finding in the shocked animals was a petechiation in the thymus. There was also in several cases evidence of damage to cerebral vessels and petechiation of the lungs, evidently due to high blood pressures reached during shock. Examination of other organs showed no gross changes.

The distribution of reduced silver in the adrenals of shocked animals suggests exhaustion of ascorbic acid in the zona fasciculata and to a limited degree in the glomerular zone as well. This is of course a consequence of the loss of ascorbic acid associated with adrenal cortical hormone production. The study of these rabbits would have been more informative had less dependence been placed on histochemical technique and a chemical assay been made of the ascorbic acid content of various tissues.

Starvation. As a comparison, three animals submitted to starvation exhibited a blood glucose depressed only to the 70 to 85 milligrams per 100 milliliters range after 16 days without food, which is far above the range of the insulinized animals. The sections made of these suggest a mobilization of ascorbic acid to the depleted cortex under the demands of shock. The adrenals of the two animals euthanized with insulin and hypoglycemic shock had slight but definite deposition of silver granules in all zones; the single animal destroyed with barbiturate produced a gland almost devoid of staining. Because of the changes due to inanition no attempt was made to identify scorbutic changes.

The purpose of the trials was to establish a technique for maintaining chronic low blood sugar levels. This intent was satisfied and with modification a second experiment was initiated.

Results of Experimental Groups

Introduction. None of the treatments used in this series of experiments could be described as seriously disturbing the physiological economy of the subjects. No frank deficiencies were induced and no toxic substances were used in the course of producing experimental changes. The advantage of such a gentle approach is manifest in the lack of gross and microscopic pathology evident in the subject animals. In all series except group I, no gross lesions could be detected upon post-mortem examination. A screening of sections of liver, small intestine and adrenal revealed no obvious pathology.

The exception, group I, has been described separately because of the difference in conduct and purpose of the experiment.

None of the animals of groups II, III and IV exhibited any visible symptoms during the course of the experiments, except two individuals of group IIIa. One of these (# 41) showed symptoms interpreted as prodromal to a hypoglycemic reaction through the last two hours of the experiment. However the terminal blood sugar level of this animal, at 53 milligrams per 100 milliliters, was above that at which animals of group I began convulsive activity, and somewhat above the range of values found in the literature. Postmortem examination showed no evidence of the rigors of hypoglycemic shock, which would further indicate that most of the lesions seen after shock are

a result of violent physical activity and possibly adrenal cortical discharge induced by very low blood sugar. The other animal (# 33) was apparently sensitive to insulin and went into shock immediately after the injection was made. Due to the circumstances it was impossible to process the tissues within the time specified for accurate analysis.

Group II - Chronic Insulin Hypoglycemia.

Weight Gain. Growth during the experimental period was considered not to be affected by the administration of insulin (Table 1). Average gain in weight for the treated group (IIa) was 571 grams and for the control group was 667 grams. This difference is due to one control animal which gained approximately twice as much as others in the group.

Table 1. Average gain of rabbits insulinized 18 days and controls

Treated (IIa)				Control (IIb)			
rabbit	initial weight	terminal weight	gain	rabbit	initial weight	terminal weight	gain
21	3028	3623	595	22	2785	3986	1201
26	2840	3392	553	23	3270	3948	678
28	2950	3462	512	24	2870	3440	570
30	3010	3480	470	25	2708	3226	518
31	3100	3750	650	27	2800	3450	650
32	2780	3428	648	29	3028	3415	387
average	2951	3522	571	average	2910	3577	667

The vast increase in food consumption of the treated animals was not as apparent in group IIa as in group Ia. The work was carried out in a cooler season but other than this no differences in handling were recognized. In fact, at certain times possibly when glucose levels were critical for a short time, the treated animals refused food for a period of a day or more. There was no evidence of respiratory or gastrointestinal infection at these times.

Adrenal Weights. Weights of adrenal glands were recorded at the termination of experiments II, III and IV. Expressed in percentage of terminal body weight there are no significant differences apparent (Table 2). From the viewpoint of this experiment this is a desirable situation, indicating that no prolonged stress has existed in the course of treatment to cause adrenal function other than normally encountered. Under these circumstances it can be assumed that no changes in ascorbic acid content other than those caused directly by the experimental procedure are to be found.

Cell Size of Zona Fasciculata. Comparison of average widths of fascicular cell cords and their nuclei to determine changes in cell size showed little variation from group to group (Table 3). The depths of the various zones of the cortex were not measured because some sections were not made through the center of the gland and zone depths were exaggerated in these instances. In addition, the differentiation of the zona fasciculata from the glomerulosa is almost an arbitrary matter

Table 2. Adrenal weights of rabbits of all groups expressed as percentage of body weight

Group										
IIa		IIb		IIIa		IIIb		IVa		IVb
No.	Chronic insulinism	No.	Control	No.	Acute insulinism	No.	Control	No.	Glucose infusion	No.	Saline infusion
21	.00228	22	.00249	46	.00400	36	.00333	37	.00269	35	.00281
26	.00327	23	.00267	41	.00242	38	.00308	40	.00290	42	.00318
28	.00274	24	.00322	39	.00217	43	.00257	44	.00334		
30	.00375	25	.00311	34	.00478	45	.00496				
31	.00303	27	.00247								
32	.00386	29	.00350								
Average	.00315		.00291		.00334		.00348		.00298		.00299

Table 3. Comparison of zona fasciculata cord width with nuclear diameter in all groups (cord width/ nuclear diameter)

Group											
IIa		IIb		IIIa		IIIb		IVa		IVb	
No.	Chronic insulinism	No.	Control	No.	Acute insulinism	No.	Control	No.	Glucose infusion	No.	Saline infusion
21	3.47	22	3.12	46	4.04	36	3.65	37	3.60	35	--
26	--	23	3.30	41	3.68	38	3.47	40	3.61	42	--
28	3.39	24	2.89	39	3.44	43	3.32	44	3.41		
30	3.23	25	3.30	34	3.10	45	3.04				
31	2.95	27	3.27								
32	3.30	29	3.86								
Average	3.26		3.29		3.56		3.39		3.54		--

unless lipid stains are used.

As an additional check against hypertrophy of the cortex sections stained with hematoxylin and eosin were examined for excessive mitotic activity. These observations bore out the findings based on adrenal weight and cell nucleus relationships which indicate no increase in activity of the gland.

Histochemistry. In comparisons of slides prepared for appraisal of ascorbic acid accumulation there is a problem of subjective influence. Slides must be compared visually several times to decide which is the most heavily stained overall, representing whole tissue accumulations, and in specific areas, representing certain functional effects. Not only must each slide be compared to each other slide on these bases, but controls and treated specimens must be handled as groups and comparisons made between groups.

In this chronic experiment on group II, the comparisons showed only slight and non-uniform differences suggesting increased amounts of ascorbic acid in the treated group. This was true of the estimation of overall staining, as well as observation of localized areas of the zona fasciculata. This zone, which is apparently responsible for glucocorticoid elaboration, comprises the bulk of the cortical substance and therefore the bulk of the entire gland, so there should be little difference between the two observations. One specimen was inadvertently delayed in fixation; the physicochemical mobility of ascorbic acid was evidenced by heavy accumulations

in the intercellular spaces and beneath the adrenal capsule, accompanied by very light staining within the cells. There is evidence of reducing substances in the medulla in some cases and the question of their mobility into cortical areas was open to consideration.

Similar examination of the liver and small intestine failed to reveal visible differences. There was an apparent nuclear concentration of reducing substance in the mucosal cells of the small intestine. It appeared that this reducing substance was removed from the cell in toto, when removed at all, so that certain cells appeared with completely stained nuclei and others had no staining.

Chemical Analysis of Tissues of Chronically Insulinized Rabbits. Chemical analysis of the tissues made possible much more precise comparisons, especially of the adrenal with its greater concentration of vitamin C. The results are directly contradictory to those of the histochemical evaluations, but at the same time should be more reliable (Table 4).

The analysis showed a marked decrease in adrenal ascorbic acid content of treated rabbits. The control group IIb averaged 179 milligrams per 100 grams of tissue and the average for rabbits treated 18 days with insulin was 120 milligrams per 100 grams. This was anticipated in projecting results of short term observations appearing in the literature (13), (36), (48). Decreases appeared in small intestine and liver on a group basis. An exception was rabbit number 31, whose liver analyzed

Table 4. Terminal tissue ascorbic acid after chronic insulinism (milligrams/ 100 grams tissue)

Group IIa				Group IIb			
No.	Adrenal	Small intestine	Liver	No.	Adrenal	Small intestine	Liver
21	147	15.1	16.3	22	162	23.7	17.4
26	106	16.7	14.8	23	152	20.1	14.8
28	155	20.1	12.2	24	181	21.4	17.1
30	92	17.3	14.2	25	190	19.6	15.7
31	120	14.7	30.5	27	197	22.4	16.3
32	103	21.5	14.2	29	186	26.3	14.6
Average	120.5	15.6	17.0		179	22.25	16.0

very high in ascorbic acid and raised the group average over that of the controls.

Blood ascorbic acid levels were generally lower in the treated animals than in the controls although values were somewhat erratic. Group IIa dropped from a pre-treatment level of 1.85 milligrams per 100 milliliters to 1.35 milligrams, a decrease of 27 percent. Group IIb, the control, dropped only 0.14 milligrams per 100 milliliters, from 2.05 to 1.91 milligrams. This seven percent lowering is accountable to normal fluctuation (Table 5).

Table 5. Initial and terminal blood glucose and ascorbic acid of rabbits under chronic insulinism (milligrams/ 100 grams tissue)

Group IIa					Group IIb				
	Glucose		Ascorbic Acid			Glucose		Ascorbic Acid	
No.	Init.	Term.	Init.	Term.	No.	Init.	Term.	Init.	Term.
21	103	40	2.1	1.67	22	110	103	2.4	1.8
26	100	43	--	1.2	23	--	140	2.4	2.6
28	122	40	2.1	1.3	24	125	129	1.6	1.2
30	97	32	1.6	1.13	25	100	93	1.87	2.2
31	122	47	1.56	1.6	27	132	104	--	1.47
32	113	54	1.87	1.2	29	93	97	2.0	2.2
Average	109.5	44	1.85	1.35		112	111	2.05	1.91

Group III - Acute Insulinism.

Adrenal Weights. No appreciable change in adrenal weight as related to body weight occurred in this group (Table 2).

Cell Size of Zona Fasciculata. No apparent differences appeared between the cell dimensions of the control and treated adrenals (Table 3).

Histochemical Preparations. Silver reduction preparations for group III were not conclusive.

Chemical Analysis of Tissues following Acute Insulinism. Group IIIa, treated with a massive dose of insulin, contained an increased amount of ascorbic acid in the adrenal and in the small intestine, and a decreased amount in the liver (Table 6).

Table 6. Terminal tissue ascorbic acid after acute insulinism (milligrams/ 100 grams tissue)

Group IIIa (insulin)				Group IIIb (control)			
No.	Adrenal	Intestines	Liver	No.	Adrenal	Intestines	Liver
46	123	19.6	13.1	36	73	14.3	13.8
41	148	15.0	15.2	38	88	19.9	9.0
39	116	16.1	14.4	43	144	25.8	12.9
34	120	19.3	25.6	45	81	19.5	16.4
Average	127	17.5	17.1		96.5	19.9	13.0

This is in contradiction to the previously mentioned observations by other investigators, and to the results of the chronic experiment just described.

The members of the control group were very low in ascorbic acid in the adrenal gland, averaging 96.5 milligrams per 100 grams. The integrity of the technique is verified in the levels found in intestine and liver which approximate those of group II. The injected rabbits show an increase to 127 milligrams per 100 grams, 30.6 milligrams higher than the controls.

These conflicting observations would leave the outcome of determinations for the control group IIIb open to some question. The results obtained were much lower than those of similar subjects or published information. In addition a distinct drop in blood ascorbic acid in the control group

suggests a dynamic situation of some nature (Table 7). The most obvious question relating to the apparent discrepancy in results would be a matter of confusion of records, in which treated and control groups were transposed. A careful recheck of procedure and records eliminated this possibility.

Table 8. Initial and terminal blood glucose and ascorbic acid and tissue ascorbic acid of rabbits made hyperglycemic by infusion of glucose

No.	Blood Glucose Init.	Blood Glucose Term.	Blood Ascorbic Acid Init.	Blood Ascorbic Acid Term.	Tissue Ascorbic Acid Adrenal	Tissue Ascorbic Acid Intestines	Tissue Ascorbic Acid Liver
Group IVa (infused glucose and saline)							
37	112	135	1.6	1.66	140	15.6	14.2
40	111	157.5	1.33	1.07	122	14.8	9.3
44	121	175	.53	1.00	66	15.0	11.4
Group IVb (infused saline)							
35	112	127	1.46	1.33	141	15.3	17.0
42	109	114	1.13	1.00	118	16.9	15.1

Group IV - Effects of Induced Hyperglycemia. In group IV three rabbits were rendered hyperglycemic by subcutaneous infusion of ten percent glucose in physiological saline, and the two control animals were similarly treated with saline alone (Table 8). The experiment was conducted as a preliminary to possible use of larger animals in similar work. Each of the

three positive animals reached a different blood level of glucose. As blood glucose increased the ascorbic acid of both the adrenal gland and the blood decreased. The lowest of the three animals in blood sugar was only slightly above normal and could be considered to have a normal distribution of ascorbic acid. The rabbit with blood glucose elevated to 175 milligrams per 100 milliliters have a very low adrenal ascorbic acid and a blood level of the vitamin which would fall in a very low normal range as judged by comparison with others in these series. Values for liver and intestine could be considered normal in each case.

DISCUSSION AND CONCLUSIONS

When insulin is carefully administered through an extended period a prolonged reduction of blood glucose values to 40 percent of normal or less may be obtained. These levels are dangerously low for the maintenance of integrity of the central nervous system, depending as it does on glucose as its sole energy source. These amounts of circulating sugar are more than adequate for maintenance of somatic activity, but because they represent at least a potential danger to the organism such sugar levels should be expected to stimulate activity of the adrenal cortex and consequent loss of ascorbic acid.

Such activity in mobilizing other sources of energy was probably not profound as can be seen in comparison of terminal

weights of rabbits so treated and their controls (Table 1). Lack of adrenal pathology or hypertrophy after lengthy hypoglycemia in the subject rabbits suggests that hypoglycemia of bovine ketosis is not primary to adrenal damage, if it is assumed that the rabbit and cow behave alike under similar hypoglycemic conditions.

The results of experiments II and III are suggestive of certain characteristics of ascorbic acid dynamics which have apparently not been completely outlined. Under a given treatment the losses and gains of tissue and blood ascorbic acid are similar. This is suggestive that ascorbic acid exists as a mobile pooled substance in the body and is drawn upon and depleted uniformly in time of need. It would also indicate that the synthesis of ascorbic acid is not a function of any single organ. The storage capacity of organs for the vitamin may be assumed to be greater than local need in order to allow equalization of vitamin levels throughout the body.

If the idea of a specific drain on the ascorbic acid pool by the adrenal cortex is not valid, an alternative must be provided. Such an assumption might place the burden of synthesis on each tissue as a highly labile reaction in which each tissue supplies its own needs as they appear. Insulin or the intracellular changes in glucose concentration which it must cause would in this case be assigned a specific effect on ascorbic acid synthesis.

The demands upon the cortices of animals in group IIIa were probably greater than in the chronic experiment because of the abrupt drop in blood sugar.

The difference in tissue and blood ascorbic acid between group IIIa and IIIb is subject to scrutiny on several counts. The pre- and post-injection levels of blood ascorbic acid changed in the controls, not in the treated animals, even though still remaining in the normal range. The control adrenals contained very low levels of ascorbic acid as compared with controls of other groups, and the liver and small intestine approach normal values. The percentage increment in adrenal, liver and blood range together however, which suggests that the determinations are valid. These data also support the contention that ascorbic acid exists as a pool, in which any change is reflected throughout the system.

The most remarkable change in experiment III is the marked increase in ascorbic acid in the treated rabbits which were subjected to massive doses of insulin. If synthesis does proceed through the initial steps of the phosphogluconate or a similar pathway, insulin may cause increased production by the same influence which causes increased fatty acid synthesis. In addition the initial effect of insulin in "driving" glucose into the cell may cause an increased ascorbic acid synthesis by mass action.

It has been demonstrated by Karg that addition of glucose in amounts sufficient to maintain normoglycemia to animals

previously administered insulin will arrest the fall of ascorbic acid (36). The study by Pancorvo dealt with human diabetics, so that the levels of insulin used can be assumed to be physiological, in comparison with the great doses used on the rabbits of group IIIa. Cotugno's experiment with insulinized humans was of necessity not profound enough to endanger the subjects (13). It remains then to find what difference in ascorbic acid concentration occurs when the low grade cortical activity accompanying mild hypoglycemia is compared with the profound activity of the cortex and synthetic tissues under deep acute hypoglycemia.

There is no explanation for the general low ascorbic acid content of this group of rabbits (group III). No known stresses were precipitated. Each animal was acclimated to handling and should have suffered a minimum of apprehension.

It has been established that the conversion of glucose to ascorbic acid is of the order of 0.3 percent in the normal rat under normal conditions (34). If this ratio is a constant at any concentration of glucose it should be reflected in changed levels of ascorbic acid as the amounts of intracellular glucose change. It seems reasonable to assume that the first stages of a gradual lowering of blood glucose would be accompanied by normal levels within the cell. Later, available carbohydrate is depleted, and insulin as added is unable to "force" enough glucose across the cell membrane, so that concentrations are lower on both sides. In this way the intra-

cellular hypoglycemic situation is provided, in which a substrate lack for both metabolism and ascorbic acid formation exists, but which may favor metabolism and diminish vitamin synthesis.

A massive increase in insulin may possibly overload the cell with glucose for a short time, providing an excess of substrate.

If, on the other hand, the synthesis of the vitamin proceeds on the basis of need, it may be assumed that even low levels of intracellular glucose would not interfere with formation. Until such a state is reached where all available glucose is utilized for the more pressing immediate needs of metabolism ascorbic acid synthesis would proceed unchanged.

A complicating factor at this point will be the efforts of the adrenal cortex to maintain the wide safety margin of circulating glucose in the face of depletion by insulin.

The results obtained from group IV do not support the idea that direct mass action regulates the synthesis, nor do they oppose it. Ascorbic acid content of the adrenal dropped abruptly in the adrenal as glucose increased, and blood, liver and small intestine showed a lesser decline. These changes did not appear in the positive controls. This observation would suggest an increase in cortical function prompted by the influx of glucose and some presumed demands which are not exerted by the mechanics of injecting large amounts of control saline. Because of the very short term of experiment IV

the mobility of the ascorbic acid pool is exceeded, and levels within the active organ are just beginning to reflect in storage tissues.

Inadequate numbers and controls emphasize that these results and conclusions of group IV must be considered only as speculation.

SUMMARY

1. A procedure has been evolved in which rabbits may be maintained for extended periods in a hypoglycemic state, with no apparent secondary influences.

2. Histochemical and chemical estimations of tissue ascorbic acid were used. The former, a subjective procedure, proved erratic and the information obtained was contradicted by the more precise and reproducible chemical analysis.

3. Eighteen days of insulinism and progressively severe hypoglycemia depleted tissue and blood ascorbic acid concentration to approximately the same degree.

4. Acute massive insulinism and hypoglycemia caused an apparent increase in blood and tissue ascorbic acid.

5. Relatively parallel increases or decreases in ascorbic acid content of tissues analyzed suggests a mobile body pool of ascorbic acid.

6. Hyperglycemia of short duration in limited tests caused a low ascorbic acid concentration, and uneven depletion of tissues

studied. This finding offers a hint as to the time factor in the mobilization scheme, and suggests that ascorbic acid synthesis is not dependent on mass of glucose available as substrate in this special case.

7. Prolonged hypoglycemia in rabbits does not result in adrenal cortical damage; on this basis it is questionable if the hypoglycemia of bovine ketosis is primary to adrenal damage found in that disease.

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GLUCOSE AND ASCORBIC ACID CONTENT OF BLOOD AND TISSUES OF NORMAL AND INSULIN INJECTED RABBITS

by

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This thesis discusses an inquiry into the effect of changes in glycemia on amounts of ascorbic acid in blood and tissues of the rabbit. The purpose of the work was to find if possible a relationship between hypoglycemia, a frequent finding in bovine ketosis and adrenal cortical function. The approach used was based on the known synthesis of ascorbic acid from glucose, and the apparent role of ascorbic acid in adrenao-cortical function.

Four groups of rabbits were used in the study. The first and fourth groups were used to explore methods for experiments described herein and future work, respectively.

Group I included six submature rabbits which were administered protamine zinc insulin three times daily in increasing doses. Dosage was increased to maintain blood sugar as low as possible without inciting convulsive activity. These animals were held in a hypoglycemic state for a maximum of 32 days for surviving animals. Most of these animals expired in hypoglycemic shock and were therefore unsuitable for the collection other than incidental data. A workable procedure for maintaining experimental hypoglycemia was evolved from this experiment.

Group II was maintained for 18 days in a hypoglycemic state by continuous administration of protamine zinc insulin. No significant changes occurred in adrenal weight, body weight gain or adrenal cell size as compared with negative controls. Histochemical evaluation of ascorbic acid distribution suggested

increased tissue ascorbic acid under insulin. Chemical analysis contradicted these subjective observations and revealed a marked decrease in ascorbic acid of the adrenal gland, small intestine, liver and blood after chronic insulin administration.

Group III was subjected to heavy acute insulinism - 50 units of protamine zinc insulin in a single dose. The animals were sacrificed at the end of seven hours and no changes in adrenal hostology appeared. Tissue chemistry revealed an increase in adrenal ascorbic acid, although both control and treated animals were lower in ascorbic acid than controls of the previous group.

Three rabbits of group IV were made hyperglycemic by subcutaneous infusion of glucose, the total administration being 150 milliliters of ten percent glucose. As blood sugar level of blood sugar.

The evidence presented suggests that ascorbic acid exists as a pool substance in various tissues and is highly mobile to areas of need.

From the lack of adreno-cortical change under hypoglycemic conditions in rabbits, a suggestion may be made that the progressive hypoglycemia in bovine ketosis is not primary to pituitary adrenal damage.