INTERACTIONS BETWEEN VITAMIN B_{12}, CORTISONE, INSULIN AND ALLOXAN DIABETES ON PROTEIN, CARBOHYDRATE AND VITAMIN B_{12} METABOLISM IN RATS

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Yu-Sheng Louise Feng

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Yu-sheng Louise Feng

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Yu-Sheng Louise Feng

AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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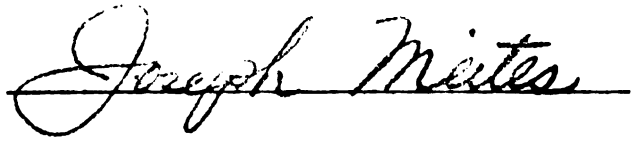
Approved by 

1. When young rats were fed a vitamin B_{12}-deficient diet, supplementation with this vitamin increased appetite and body weight gains, slightly increased blood glucose, greatly increased glucose tolerance, but slightly decreased urinary nitrogen excretion. When one to four mg. of cortisone acetate daily were injected into vitamin B_{12}-deficient rats, there was a progressive increase in urinary nitrogen, increased hyperglycemia and glucosuria, decreased glucose tolerance, reduced body weight gains and decreased appetite. When 200 mcg. of vitamin B_{12} per kilogram of diet was fed to cortisone-injected rats, and they were permitted to eat ad libitum, increases in urinary nitrogen losses were largely prevented, the hyperglycemia and glucosuria were reduced, glucose tolerance was increased and body growth was increased. Vitamin B_{12} was ineffective in these respects when food intake was restricted to that of animals receiving cortisone without vitamin B_{12}. It is concluded that large doses of vitamin B_{12} can partially counteract the protein catabolic actions of cortisone by increasing appetite, increasing the availability and utilization of carbohydrate by the organism and reducing gluconeogenesis from protein.

2. Large doses of cortisone partially interfered with the favorable action of vitamin B_{12} in increasing the efficiency of food utilization for body growth. This was accompanied by hyperglycemia and glucosuria, and was related

to increased insulin resistance. Less carbohydrate was therefore left available for transformation into body weight gains (probably fat).

3. Alloxan-diabetes reduced body growth and the feed/gain ratio on the vitamin B_{12}-deficient but not on the vitamin B_{12}-adequate diet. In the latter rats there was much higher blood glucose, more urinary glucose, increased glucose tolerance but about the same urinary nitrogen losses as in the former animals. It is concluded that vitamin B_{12} can act independently of insulin insofar as its effects on glucose utilization and body growth are concerned.

4. Single injections of insulin (2 units in most cases) were much more effective in reducing blood glucose in normal, alloxan-diabetic and cortisone-treated rats on a vitamin B_{12}-adequate than on a vitamin B_{12}-deficient diet. This indicates that an ample supply of vitamin B_{12} is essential for maximum insulin action . By far the greatest resistance to insulin was found in the cortisone-treated rats on the vitamin B_{12}-deficient diet, confirming the findings that cortisone increases insulin resistance.

5. (a) Injections of large doses of cortisone (2 to 4 mg. daily) increased the urinary excretion of radioactive vitamin B_{12}, particularly in rats fed a vitamin B_{12}-deficient diet. On a diet meeting only normal requirements for vitamin B_{12} (20 mcg./kilogram), cortisone did not increase urinary

vitamin B_{12} until 4 mg. were injected daily. In general, the amounts of radioactive vitamin B_{12} lose in the urine were shown to be directly related to the dose of cortisone administered.

(b) Intraperitoneal injections of 750 mg. of glucose did not change urinary losses of vitamin B_{12} in alloxanized rats fed either a vitamin B_{12}-adequate or -deficient diet. Apparently blood glucose was already being used to the maximum extent possible in these rats.

(c) In normal and cortisone-treated rats on a vitamin B_{12}-adequate but not on a vitamin B_{12}-deficient diet, intraperitoneal injections of glucose decreased the loss of urinary vitamin B_{12}. This is believed to reflect greater glucose utilization in the former animals.

(d) Insulin injections (3 injections of 0.5 unit each in 24 hours) greatly reduced urinary radioactive vitamin B_{12} losses in normal, alloxanized and cortisone-treated rats whether on a vitamin B_{12}-adequate or -deficient diet. This is believed to reflect greater glucose utilization in these animals. The decreases in urinary vitamin B_{12} were less on the vitamin-deficient diet, particularly in the cortisone-treated animals, and is believed to reflect the reduced effectiveness of insulin on glucose utilization in these rats.

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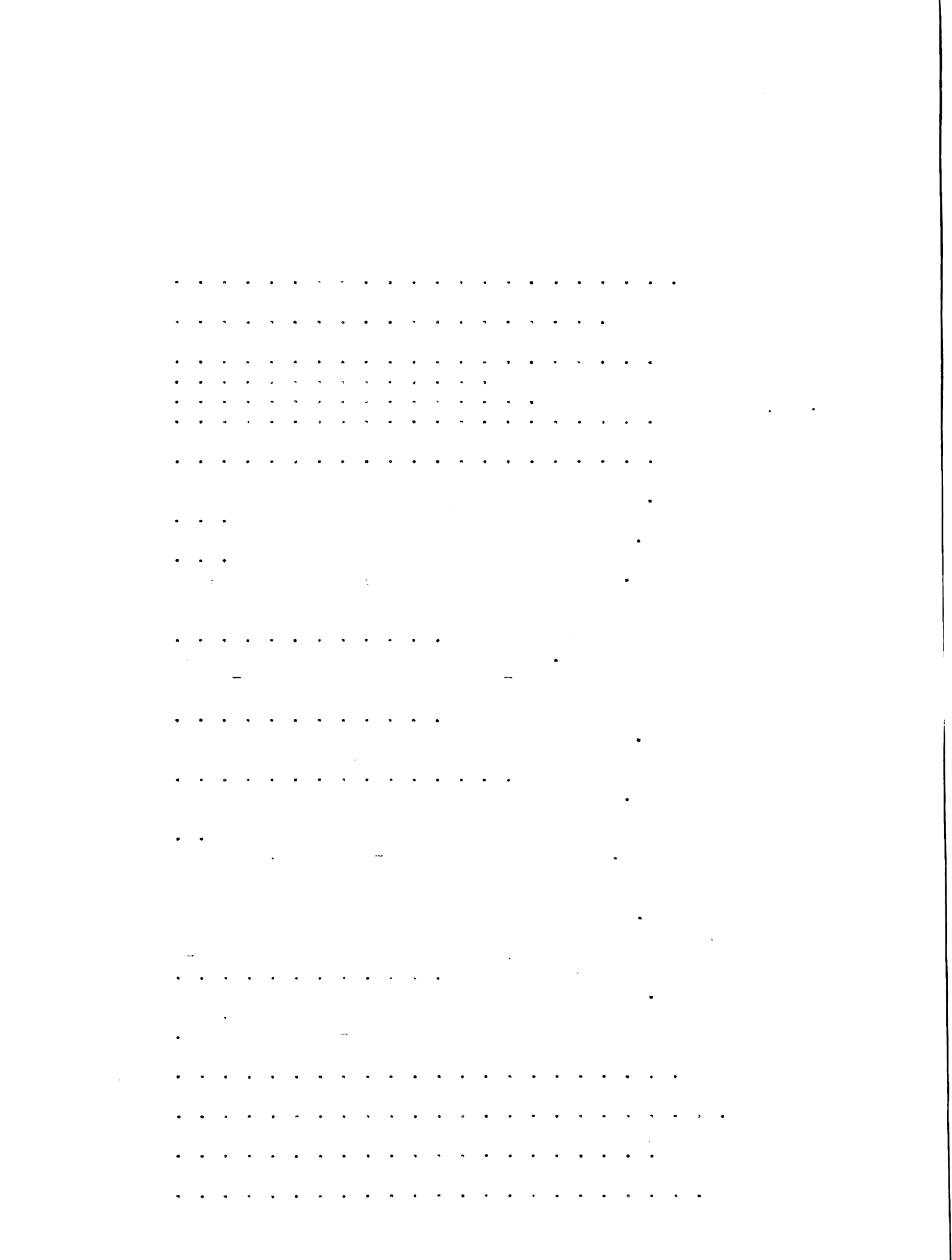

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INTRODUCTION

Large doses of cortisone have been shown to produce catabolic effects characterized by reductions in body, hair and thymus growth and by increased gluconeogenesis from protein. There is also considerable evidence that large doses of cortisone stimulate pancreatic islets function (Baker, 1952; Franckson et al. 1953), but at the same time interfere with the action of insulin on carbohydrate utilization (Ingle et al. 1945; Franckson et al. 1953). Vitamin B_{12} has recently been demonstrated to favor transformation of carbohydrate into fat (Chow et al. 1951, 1952), a function which is also characteristic of insulin. The foregoing suggests that the action of cortisone, insulin and vitamin B_{12} may be interdependent insofar as their effects on carbohydrate and protein metabolism are concerned.

In a series of reports Meites (1951, 1952a, 1952b) observed that large doses of vitamin B_{12} partially counteracted the inhibitory effects of large doses of cortisone on body, hair and thymus growth in young rats. These beneficial effects were invariably accompanied by increased food intake and by greater efficiency in converting food into body weight gains, but at the same time cortisone prevented vitamin B_{12} from exerting its effects in full. It was therefore of interest to attempt to discover how these interactions

between cortisone and vitamin B_{12} were produced, and to determine what the role of insulin might be in this process. Specifically, this thesis will deal principally with the following questions.

1. By what means do large doses of vitamin B_{12} partially counteract some of the catabolic actions of cortisone? Does vitamin B_{12} prevent increased gluconeogenesis from protein? If so, through what mechanism?
2. How do large doses of cortisone partially interfere with the ability of vitamin B_{12} to transform food into body weight gains (i.e., transform carbohydrate into fat)?
3. Does vitamin B_{12} require insulin to increase food intake, body growth and glucose utilization or can it function independently of insulin?
4. Does insulin require vitamin B_{12} for its action on carbohydrate metabolism or is its action independent of vitamin B_{12}?
5. What are the reactions to glucose tolerance tests of normal, alloxan-diabetic and cortisone-treated rats on a vitamin B_{12}-deficient or -adequate diet? This should indicate to what extent these rats can utilize glucose.
6. What are the effects of cortisone, alloxan-diabetes, insulin or glucose injections on vitamin B_{12} requirements, as measured by urinary excretion of injected radioactive vitamin B_{12}?

Of course complete answers to the above questions were not forthcoming in the research reported here, but it is believed that some of the interrelationships between cortisone, insulin and vitamin B_{12} have been clarified. Several if not many additional questions have arisen as a result of the findings recorded in this thesis, and only further investigation can be expected to resolve them.

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LITERATURE REVIEW

Introduction

Inasmuch as this thesis deals with interactions between vitamin B_{12}, cortisone, insulin and diabetes as related to protein, carbohydrate and vitamin B_{12} metabolism, it is pertinent to briefly review some of the salient actions of the former on the latter. The writer found it necessary to select from a vast literature, and for the most part the articles reviewed here were chosen because of their direct bearing on the thesis problem.

Adrenal Cortical Hormones

Effects of cortisone and ACTH on body, hair and thymus growth

The adrenal cortical hormones play an important part in the maintenance of normal growth processes. This function appears to depend primarily upon the effects exerted by the adrenal cortical hormones on protein and carbohydrate metabolism. Protein synthesis is retarded in adrenalectomized animals, but is not altered further during stress. Ingle (1949) found that during severe stress induced by bone fracture or burns in the adrenalectomized rats, the breakdown of protein was not accelerated as it was in normal animals. Injection of high doses of ACTH or cortisone

causes impairment of growth because of inhibition of new protein formation and protein catabolism. The growth-inhibiting potency of ACTH or cortisone parallels the magnitude of the negative nitrogen balance (Ingle, 1946).

Administration of ACTH (Baker et al. 1948) and cortisone (Winter et al. 1950) to rats suppressed growth of hair. Ingle (1949) stated that the prolonged local application of adrenal cortical extract or of cortisone to the skin of rats reduced the cellularity of the dermis. Dougherty and White (1945) found that treatment with ACTH or cortisone induced involution of the thymus, lymph-nodes and spleen. They stated that lymphocytes underwent lysis within a few hours after treatment. Baker et al. (1951) also found that new formation of lymphocytes was suppressed as indicated by a reduction in mitosis and immature cells, and destruction of reticular tissue cells from which lymphocytes originated.

Effects of cortisone and ACTH on carbohydrate and protein metabolism

The possible relationship between adrenal cortical function and carbohydrate metabolism was first appreciated by Britton (1932). In adrenal cortical insufficiency the carbohydrate content of the blood and tissues was decreased below normal. In contrast, administration of cortisone or ACTH has been shown to increase liver glycogen, and produce hyperglycemia and glucosuria. Long, Katzin and Fry (1940) found that administration of adrenal cortical extract or

crystalline carbohydrate-active adrenal steroids to fasted adrenalectomized or to normal or hypophysectomized animals resulted in a ten-to-forty-fold increase in liver glycogen, and increases in blood glucose and urinary nitrogen excretion. Ingle (1940) noted a diabetogenic effect of cortisone in partially depancreatized and normal rats as indicated by hyperglycemia, glucosuria and increased excretion of non-protein nitrogen.

Large doses of either ACTH or of cortisone have been observed to produce a negative nitrogen balance in laboratory animals and in man (Long et al. 1940; Ingle, 1940). Engel (1951) stated that it was probable that the adrenal cortical hormones acted predominately at the level of whole protein rather than at some intermediary stage of nitrogen metabolism. Support for this statement comes from the work of Ingle et al. (1950) who found that cortisone accelerated the rise of amino acids in the blood of liverless rats.

The classical work of Long, Katzin and Fry (1940) formed the basis for the present knowledge of the effects of adrenal cortical hormones and ACTH upon carbohydrate and protein metabolism. The observation that the rise in glucose in the blood and tissue was paralleled by a rise in the excretion of non-protein nitrogen led tjem to believe that the hormones possibly stimulated gluconeogenesis from tissue proteins. Adrenalectomized animals showed an inadequate blood glucose level when exposed to circumstances demanding

an increased rate of protein metabolism. Ingle (1945) induced severe glucosuria in normal rats by administering cortisone, but elieved that the extent of the glucosuria was too great to be completely accounted for by gluconeogenesis from protein. Albright (1943) stated that cortisone inhibited the synthesis of protein rather than accelerated protein catabolism. The balance between the rate of breakdown and resynthesis of tissues could be shifted in the direction of tissue depletion by either stimulation of catabolism or inhibition of anabolism. Hoberman (1950) and Clark (1953) reported evidence for both an acceleration in protein catabolism and an inhibition of anabolism in experiments involving the use of N^{15}-labeled glycine in rats given either cortisone or ACTH.

Engel (1949) reviewed the evidence that carbohydrate and other foodstuffs could decrease the catabolic effect of the adrenal cortical hormones and suggested that the hyperphagia which frequently occurred during treatment with ACTH or cortisone represented a homeostatic response by the body which tended to sustain nitrogen balance. Long, Katzin and Fry (1940) and Engel (1949) noted that administration of carbohydrate to cortisone treated rats prevented the protein-catabolic effect of the latter. This suggests that the adrenal steroids may also act directly on carbohydrate. As will be seen later, these observations have a direct bearing on the results reported here with vitamin B_{12} in cortisone-treated rats.

Relation of cortisone and ACTH to function of the pancreas

Since the level of blood sugar appears to control the production of insulin by the beta cells of the islets of Langerhans, Jensen (1948) stated that any agent which induced hyperglycemia could be expected to stimulate this gland indirectly. Baker et al. (1952) found that ACTH cuased hypertrophy, degranulation and an increase in number of beta cells in the islets of Langerhans in rats. Kobernick and More (1950) and Franckson et al. (1953) found hydropic degeneration of the islets of rabbit and rat after long-term treatment with cortisone.

Ingle et al. (1945) reported that normal rats made diabetic by either cortisone or hydrocortisone were highly resistant to insulin. Adrenal steroid diabetes with insulin resistance was described by Sprague (1950) in a patient with Cushing's syndrome who excreted large amounts of hydrocortisone in the urine. Franckson et al. (1953) found that cortisone administration was followed by a transitory diabetes in rats, characterized by hyperglycemia, lessened glucose tolerance and marked resistance to insulin. They concluded that steroid diabetes was due to reduced glucose utilization because of inhibition of insulin activity. In confirmation of these results Boutwell and Chiang (1954) reported that large doses of cortisone depressed oxidation of glucose in the mouse.

Diabetes and Insulin

Effects of insulin on carbohydrate, protein and fat metabolism

The primary manifestation of insulin action *in vivo* is a lowering of blood sugar level. This may be elicited by decreasing the production of blood sugar by the liver or by increasing the utilization of glucose in the organs and tissues. The energy provided by the reactions of intermediary metabolism is used by the cell for the performance of work, including growth and reproduction, as well as mechanical work such as muscular contraction. The overall process involves the oxidation of foodstuffs to carbon dioxide and water. The energy so generated is not released all at once as it is when sugar is burned in air. Instead, the original assimilated foodstuff molecules undergo a series of intermediate reactions. In each step, energy is absorbed or liberated by the synthesis or cleavage of the chemical bonds present in the intermediate compounds. Insulin is the major hormone in the body that is able to accelerate the removal of glucose from the blood, as well as its transformation and ultimate utilization by the tissues. Since skeletal muscles are the largest organs concerned with glucose utilization, it is the effect of insulin on this tissue that has been most studied although its effects on glucose metabolism are concerned with other organs, particularly the liver.

Long (1954) stated that insulin either directly or

indirectly accelerates the rate of glucose utilization by three major metabolic pathways: (1) polymerization of glucose to glycogen both in muscles and liver; (2) conversion to fatty acids both in liver and in adipose tissues; and (3) an ultimate increase in the proportion of glucose or glycogen that is oxidized to carbon dioxide and water.

Stadie, Haugaard and Marsh (1952) studied the isolated rat diaphragm and found that the effect of insulin increased with increasing glucose concentration. Bouckaert and de Duve (1947) measured quantitatively the amount of glucose which disappeared in the liver and in the peripheral tissues under the action of insulin. By comparing normal and hepatectomized animals with respect to the amount of glucose needed to maintain a constant level of blood sugar, they found that liver accounted for a large fraction of total glucose utilization. They concluded that insulin promoted the net uptake of glucose by the liver, since hepatectomy greatly diminished the amount of glucose necessary to maintain the blood sugar level after a large dose of insulin. Bouckaert *et al.* (1947) and Wick *et al.* (1951) worked on intact and eviscerated animals and concluded that the primary physiological effect of insulin in lowering blood sugar was its increase in utilization of glucose in the organs and tissues of the body, and in decreasing the net production of glucose by the liver.

In the absence of insulin the diabetic organism excretes

abnormally large amounts of nitrogen in the urine (Luck *et al.* 1933; Duncan *et al.* 1942; Macleod, 1926). This indicates that insulin must act to inhibit protein catabolism at some point. Bach and Holmes (1937), using liver slices *in vitro*, showed that insulin inhibited the deamination of amino acids. This was accompanied by a decreased rate of appearance of carbohydrate, leading to the conclusion that insulin inhibited gluconeogenesis from amino acids and therefore from protein. This nitrogenous sparing effect of insulin was further demonstrated by Gaebler and co-workers (1945) who found that whereas extracts of the anterior pituitary administered to normal animals resulted in nitrogen retention, the same treatment in diabetic animals caused an increased nitrogen excretion. Lotspeich (1949) suggested that insulin promoted protein synthesis *in vivo*. This view was based on results which indicated that insulin accelerated the disappearance of amino acids from the blood stream in about the same proportion as these amino acids occurred in muscle protein. More recently Best (1952) showed that it was possible to induce the hypophysectomized rat to grow by treatment with insulin when food intake was not controlled. He concluded that insulin enhanced the appetite and accelerated protein anabolism.

There are many indications that insulin influences the metabolism of fat. Stetton and Boxer (1944) demonstrated that one of the main defects in diabetes was an inability to

synthesize fat. Liver slices from diabetic rats were found to have a greatly diminished ability to synthesize fat from acetate (Brady et al. 1951) or glucose (Chernick et al. 1950). On the other hand, Brady et al. (1951) found that insulin accelerated the incorporation of C^{14}-labeled acetate into long-chain fatty acids.

Control of insulin secretion

Since insulin secretion is stimulated by an increase in blood glucose, any factor which serves to increase blood sugar will increase the function of the pancreas. Anderson, Lindner and Sutton (1947) studied the insulin content of perfusate coming from the isolated pancreas, using the hypophysectomized, adrenal-demedullated, alloxan-diabetic rat. They reported an increase in the output of insulin by the isolated pancreas when the glucose concentration of the perfusing fluid was elevated. Soskin and Allweiss (1934) found that it required a constant injection of insulin to maintain a normal blood sugar level in the depancreatized dog.

Two hormones which are concerned indirectly with the secretion of insulin are growth hormone from the anterior hypophysis and glucagon from the pancreas. The major effect of growth hormone appears to be a suppression of carbohydrate utilization by the peripheral tissues, particularly by skeletal muscles (Long, 1954). Anderson and Long (1947) also demonstrated that growth hormone inhibited insulin secretion

by the isolated perfused pancreas. Krahl and Park (1948) reported that growth hormone injections depressed the glucose uptake of the rat diaphragm. It was concluded that growth hormone inhibited the utilization of glucose and diminished the response to insulin.

In the year following the isolation of an hypoglycemic factor from the pancreas by Banting, Best and Collip (1922), Kimball and Murlin (1923) reported a transient hyperglycemia following the administration of crude extract of pancreas containing insulin, and named the hyperglycemic factor or factors which they detected "glucagon". Sinn and Behrens (1953) reported that glucagon is a protein and can be crystallized from pancreas. Under continuous intravenous infusion of glucagon accompanied by insulin in amounts which alone would produce hypoglycemia, glucagon was found to be capable of preventing hypoglycemia and even of maintaining an hyperglycemia for at least six hours (de Duve, Hers, Bouckaert, 1946; Weisberg, Caren, Huddlestun and Levine, 1949; Tyberghein, 1952, 1953; Myers et al. 1953). Bornstein, Reid and Goring (1951) administered growth hormone to rats and cats and injected portal blood from these treated animals into adrenalectomized-hypophysectomized, alloxan-diabetic rats. This procedure elicited hyperglycemia in the test animals, but not when blood from a peripheral vein was injected. Support of their observations was provided by Foa

and co-workers (1953) who reported that following injection of purified growth hormone in donor dogs, an hyperglycemic factor appeared in blood from the pancreas but not from peripheral blood. It was concluded that growth hormone administration caused the liberation from the pancreas of a hyperglycemic factor, presumably "glucagon", which was rapidly destroyed in normal blood.

Relation of insulin and diabetes to B-vitamins

The problem of maintaining proper nutrition in controlled diabetes appears to be a quantitative relation between the amount of insulin and the amount of carbohydrate in the diet which can be utilized. With decreased utilization of carbohydrate in insulin-deficient individuals, there apparently is a decreased need for the accessory factors associated with carbohydrate metabolism. Thiamin, niacin, and perhaps pantothenic acid act as co-enzymes in carbohydrate oxidation systems. According to Samuels (1948), the need for these vitamins is reduced in the diabetic just as it is in the normal animal on a high fat, low carbohydrate diet. On administration of insulin the intake of B-vitamins becomes high, since the tissue concentrations have to be restored as well as provide for the increased utilization of carbohydrate.

Not only is the need for B-vitamins dependent upon the available insulin, but the effectiveness of insulin appears to be influenced by any deficiency of these factors. Martin

(1937) found that depancreatized dogs on a vitamin B-deficient diet became resistant to insulin. Elsom, Lukens, Montgomery and Jones (1940) reported a progressive decrease in the response to insulin as a deficiency of the B-complex was produced experimentally in a woman. On feeding riboflavin and thiamin, this subject became abnormally sensitive to insulin. Biskind (1945) reported that vitamin therapy was effective in decreasing the hormone requirement in insulin-resistant diabetes. Best et al. (1939) found that in thiamin deficiency reduction of insulin production occurred. Apparently this was due to the inanition rather than to thiamin lack per se, since animals limited in food intake but receiving ample thiamin showed a similar drop in insulin content. Since there is good evidence that vitamin B_{12} is necessary for normal carbohydrate metabolism (Chow et al. 1952, 1953, 1954), there is a possibility that insulin may increase the need for this vitamin. Evidence to support this view will be presented in the experimental data.

Vitamin B_{12}

Effects on protein metabolism

Cary and Hartman (1943-1947) showed that an "animal protein factor" was present in natural materials such as whole milk, cheese and liver extract but absent in yeast and vegetable foods. They also noted that extraction with hot alcohol removed this factor from commercial casein and

that the deficiency could be made more acute in rats by adding hot alcohol-extracted casein to a soybean meal diet. They claimed that an "animal protein factor" was needed for the metabolism of protein at some stage, since a deficiency of this factor was accentuated by raising protein levels in the diet. They found it possible to concentrate this factor from liver extracts. Ott, Rickes and Wood (1948) reported that crystalline vitamin B_{12} had "animal protein factor" activity in chicks on all-vegetable diets, and subsequently it was believed that these two factors were the same. However, there is some doubt that all "animal protein factors" contain only vitamin B_{12}.

Hsu and Combs (1952) claimed that a vitamin B_{12} deficiency in chicks increased the blood levels of non-protein nitrogen, amino nitrogen, urea nitrogen, creatinine, and glucose as compared to chicks receiving crystalline vitamin B_{12}. The level of uric acid was not consistently affected. It was suggested that vitamin B_{12} is involved in nitrogen metabolism in the chick. Cheng and Thomas (1952) attempted to determine whether vitamin B_{12} played a part in utilization of protein for the growth of animal tissues. Under rather rigid conditions of vitamin B_{12} depletion, it was demonstrated that vitamin B_{12} injections increased the utilization of protein as judged by its capacity to increase nitrogen retention in rats. The evidence indicated that vitamin B_{12} aided in the conversion of the amino acid homocystine to methionine. In

the case of soybean protein, which is low in methionine, vitamin B_{12} helped the animals more completely to utilize this food. Charkey et al. (1953) investigated the possibility that vitamin B_{12} may enhance utilization of amino acids in chicks. They found that both vitamin B_{12} and "animal protein factor" promoted growth and increased blood levels of arginine and methionine, but had no effect on blood tryptophane, lysine and histidine.

Effects of carbohydrate and fat metabolism

Animal responses to several of the B-vitamins can be altered by changing the dietary compositions with regard to the three major foodstuffs: fat, carbohydrate and protein. Information gained in this type of study has been of prime importance in elucidating the mechanism of action of these vitamins. It was therefore considered worthwhile by Bosshardt (1950) to apply this method of nutritional investigation to vitamin B_{12}. Variations in dietary composition with regard to fat, carbohydrate and protein were shown to have an influence on vitamin B_{12} needs in the growing mouse. A decrease in the fat level of the diet with a corresponding increase in carbohydrate intensified the growth retardation due to a deficiency of vitamin B_{12}. This growth retardation was partially corrected by the feeding of fat or administration of vitamin B_{12}, and was completely overcome by administration of both fat and vitamin B_{12}.

Chow *et al.* (1951, 1952) reported that when rats receiving supplementary vitamin B_{12} by injection were pair-fed to controls, no growth stimulation followed administration of vitamin B_{12}; but if the controls were force-fed to match the intakes of the supplemented animals eating *ad libitum*, the former animals gained much more weight during the experimental period. However, under neither condition was any difference in nitrogen balance detectable. They concluded that vitamin B_{12} was without effect on the retention of nitrogen over a broad range of nitrogen and caloric intakes. By analyzing carcass composition they showed that vitamin B_{12}-deficient weanling rats had a high water and low fat content. Under the influence of vitamin B_{12}, these values returned to normal levels quite characteristic of those recorded for healthy animals on good stock diets. But no effect was seen on the proportion of protein in the carcass. They claimed that vitamin B_{12} was, in some as yet unknown manner, involved in transformation of carbohydrate to fat, and seemed to play no direct part in protein metabolism. They also concluded that the role of vitamin B_{12} appeared to be regulatory, since animals receiving abundant quantities did not become obese but tended to revert in carcass composition to generally accepted normal values. In confirmation of the above, Arnrich *et al.* (1952) reported that when vitamin B_{12} or aureomycin or both were added to a purified diet and fed to dogs, nitrogen metabolism was unchanged but the dogs gained more weight.

These dogs had greater carcass fat and more fat-rich adipose tissue. They concluded that the increased weight gains were due to increased fat deposition.

Ling and Chow (1954) studied the effects of vitamin B_{12} on carbohydrate and fat metabolism by glucose tolerance tests and by estimation of the phospholipid content of blood and tissues. It was found that a vitamin B_{12} deficiency prevented normal carbohydrate utilization, as indicated by an abnormally high blood glucose level following intravenous injections of glucose. The blood glucose levels returned to normal at a much slower rate than in vitamin-adequate animals. They concluded that vitamin B_{12}-deficient rats lost part of their ability to transform carbohydrate to fat. Their data showed further that abnormally small amounts of phospholipids were found in tissues of vitamin B_{12}-deficient rats and in blood of patients with pernicious anemia in relapse. Administration of vitamin B_{12} to the above resulted in marked increases in the phospholipid content of the blood and tissues.

Vitamin B_{12}-deficient rats also showed a marked diminution in levels of soluble sulfhydryl compounds in blood, which rose with the administration of this vitamin (Ling and Chow, 1954). It was suggested that this was due to the change in concentration of glutathione. This was corroborated by Register (1954) who also found a marked decrease in the levels of liver and blood sulfhydryl groups when rats

were fed a soybean ration deficient in vitamin B_{12}. It was agreed that this was due primarily to a glutathione deficiency. Administration of glutathione or vitamin B_{12} lowered the blood glucose levels of rats with hyperglycemia induced by a high carbohydrate, low fat diet or by glucose injections.

Interactions between vitamin B_{12} and adrenal cortical hormones

Meites (1951, 1952a, 1952b) reported that cortisone depressed body, hair and thymus growth in growing rats fed a soybean meal or a semi-synthetic diet. These effects were completely or partially prevented by incorporating 200 mcg of vitamin B_{12} per kilogram of diet or 0.005 percent aureomycin. It was also found that vitamin B_{12} was more effective than aureomycin, and that the combination of the two substances was more effective than either alone. The favorable action of the vitamin and antibiotic were accompanied by an increase in food consumption and greater efficiency in converting food into body weight gains, although the latter effect was reduced from that found in non-cortisone treated animals. It was concluded that large doses of cortisone increased requirements for vitamin B_{12} in the young rat.

Rupp and Paschkis (1951) reported that when vitamin B_{12} was administered to force-fed hyperthyroid rats on a constant food intake, the weight loss was identical with that of hyperthyroid animals not receiving vitamin B_{12}. However, vitamin B_{12} decreased the loss of nitrogen resulting from

the catabolic action of thyroid. They also reported (1953) that vitamin B_{12} failed to influence cortisone-induced protein catabolism in rats when food intake was limited. Ershoff (1951, 1953) reported that liver residue contained a factor other than vitamin B_{12} which counteracted the growth-retarding effect of thyroid powder or cortisone fed to young rats.

Wahlstrom and Johnson (1951) reported that large doses of cortisone injected into baby pigs on a vitamin B_{12}-deficient diet increased the urinary excretion of vitamin B_{12}, as determined by microbiological assay. Chow et al. (1953) performed the same type of experiment in rats. They reported that vitamin B_{12} activity in 24-hour urine specimens of the cortisone-treated animals was twice that of control animals, as measured either by microbiological assay or by concentration of radioactive vitamin B_{12}. The tissue analysis demonstrated that in each instance the organs of the cortisone-treated animals retained less radioactive vitamin B_{12} than the controls.

Interactions between vitamin B_{12} and diabetes

Harte, Chow and Barrows (1953) observed a marked retention of radioactivity in the pancreas following injection of radioactive vitamin B_{12} into rats, and suggested a possible role for vitamin B_{12} in pancreatic diseases which might be manifested by an abnormal excretion of this vitamin. Further

studies by Chow *et al.* (1953) indicated a possible correlation between the urinary excretion of administered vitamin B_{12} and diabetic retinopathy (sometimes seen in advanced stages of diabetes). The diabetics with retinopathy excreted much more radioactive vitamin B_{12} in the urine than diabetics without retinopathy. Since the dietary history of these patients was not given, it is difficult for the writer to determine to what extent the two forms of diabetes influenced vitamin B_{12} retention.

EXPERIMENTAL

Experiment I. Prevention by Vitamin B_{12} of Protein Catabolic Action of Cortisone

Purpose

Since it had been demonstrated that large doses of vitamin B_{12} could partially counteract certain catabolic effects of cortisone in young rats (Meites, 1951, 1952, 1953), it was of interest to determine whether this was mediated to any extent by preventing excessive protein breakdown. The effect of cortisone on urinary nitrogen losses was determined on rats fed diet deficient or excessive in vitamin B_{12}.

Methods

Forty Carworth male weanling rats were fed a stock soybean meal diet deficient in vitamin B_{12} for a preliminary period of 30 days. The composition of the diet is described in the appendix. Water and food were available at all times. The rats were housed in metal cages, 10 to a cage, at a mean room temperature of $76^{\circ} \pm 1^{\circ}$ F. Artificial light was available daily from 7:30 AM to 9:30 PM. Twenty-four hour urine specimens were collected every ten days and total urinary nitrogen was determined by a standard micro-Kjeldahl procedure (Hawk *et al.* 1951), described in the appendix.

After the 30-day depletion period, the rats were divided into four uniform groups and were treated as follows for an additional 30 days:

Group 1. No vitamin B_{12}

2. 200 mcg. vitamin B_{12}/kilogram of diet

3. Cortisone

4. Cortisone + vitamin B_{12}

Groups 3 and 4 received 1 mg. of cortisone acetate (from here on referred to as cortisone) daily by subcutaneous injection for the first 10 days, 2 mg. daily for the second 10 days and 4 mg. daily for the third 10 days. A total of 200 mcg. of crystalline vitamin B_{12} was mixed with one kilogram of the stock diet. This amount of the vitamin represents approximately ten times the normal requirement (Stokstad et al. 1949; Zucker et al. 1950). Body weight and food consumption were measured every two days. Urinary nitrogen was determined every five days. For urine collection, five rats were placed in a single metabolism cage in which water was available at all times and food was present in non-scatter metal feeders. The animals were kept in the cage for 24 hours and the urine was collected in flasks containing one gram of citric acid as a preservative. All the specimens were placed in a refrigerator at the end of 24 hours.

In this and all subsequent experiments the standard

error of the mean was determined by the following formula:

$$SEM = \frac{d^2}{n(n-1)}$$

Results

It can be seen in Fig. 1 that the 40 rats averaged 145 grams each at the end of the 30-day depletion period. The rats which received vitamin B_{12} (Group 2) reached an average body weight of 288.2 grams each as compared to the vitamin-deficient rats in Group 1 which averaged only 205.8 grams each. The rats in Group 3 which were treated with 1 mg. of cortisone daily for 10 days without vitamin B_{12}, gained only 3 grams in body weight; when 2 mg. of cortisone were injected daily, growth was completely suppressed; and 4 mg. of cortisone daily resulted in loss of body weight. When vitamin B_{12} was added to the ration (Group 4), cortisone did not prevent growth although the body weight did not reach the level of the rats given vitamin B_{12} alone (Group 2).

The rats fed vitamin B_{12} (Groups 2 and 4) had the highest food intake and efficiency of utilization for body growth. The total food intake in Group 2 was 730 grams while in the cortisone-treated vitamin B_{12}-deficient rats (Group 3), the total food intake was only 428.8 grams. Food intake was increased in Group 4 when vitamin B_{12} was added to the diet, but the efficiency of food utilization was well below the two groups which were not treated with cortisone (Groups 1 and 2).

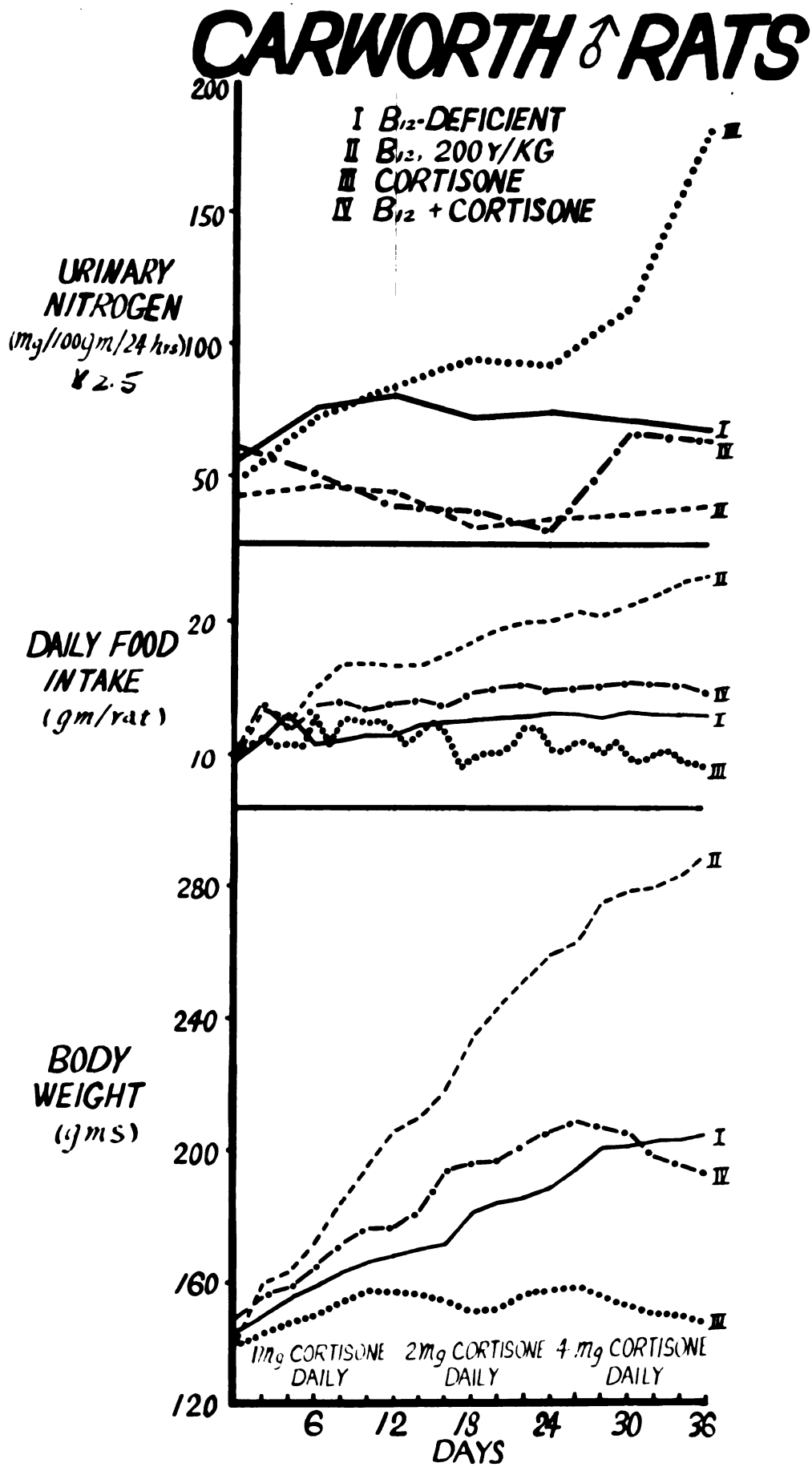


Fig. I Effects of vitamin B12 and cortisone on urinany nitrogen and food intake.

The average daily excretion of urinary nitrogen per 100 grams of body weight (Table I) appeared to be greater in the vitamin B_{12}-deficient animals (Group 1) than in the vitamin B_{12}-adequate animals (Group 2). However, there was no increase in urinary nitrogen after vitamin B_{12} administration to Group 2. Cortisone progressively increased the urinary nitrogen excretion in the vitamin B_{12}-deficient animals (Group 3). After the third 10-day period of treatment with cortisone, the nitrogen excretion per 100 grams body weight per day was almost three times as high as in the pre-treatment period. Vitamin B_{12} largely prevented this increase of urinary nitrogen excretion induced by cortisone (Group 4).

Conclusions

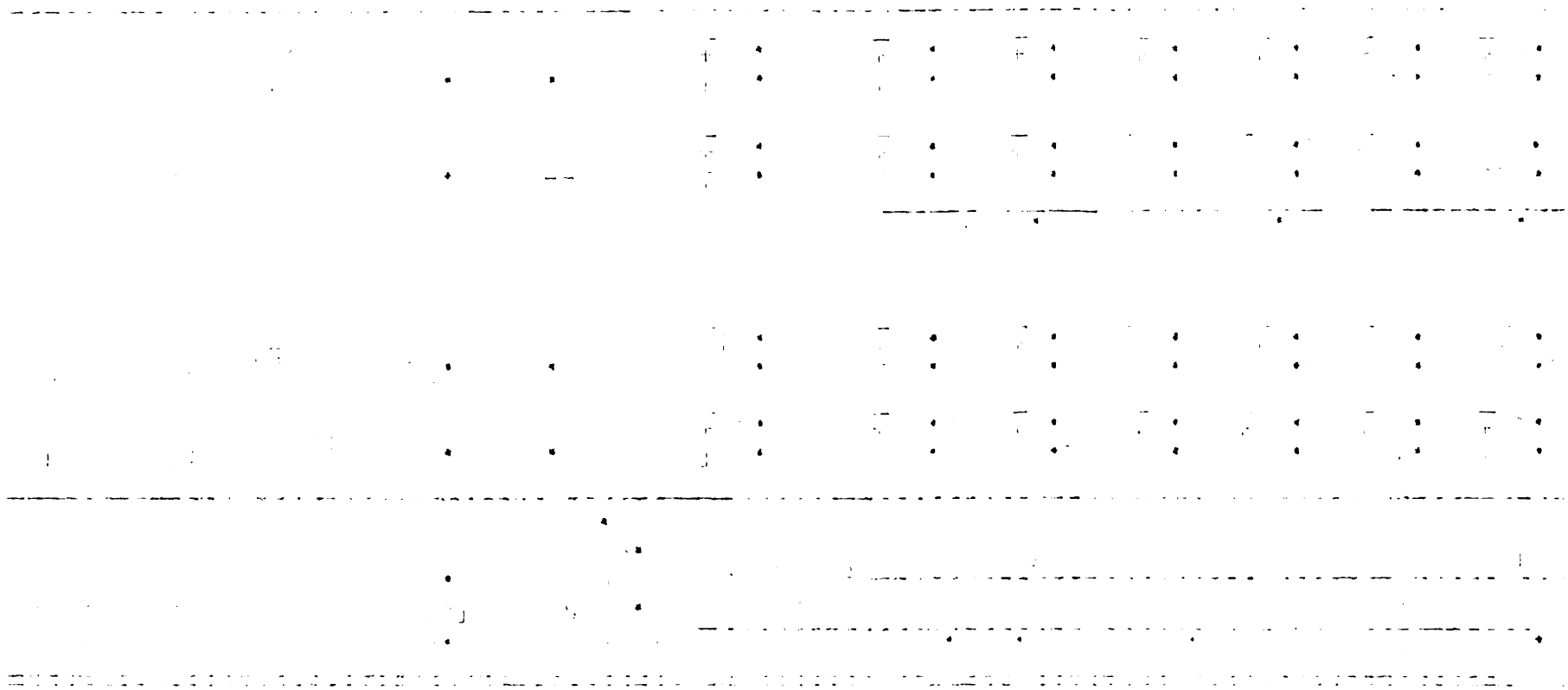
Vitamin B_{12} largely prevented these protein catabolic actions of cortisone under *ad libitum* feeding. Therefore it may be concluded that vitamin B_{12} can prevent increased urinary nitrogen, probably by increasing appetite and thereby enhancing the availability of carbohydrate to the organism. This is in agreement with the work of Long *et al.* (1940) and Engel (1949) who showed that administration of large amounts of carbohydrate together with adrenal cortical extract to rats prevented the protein catabolic effects of the latter. In addition, large doses of cortisone markedly inhibited the ability of vitamin B_{12} to transform food into body weight

TABLE I

EFFECTS OF VITAMIN B_{12} AND CORTISONE ON URINARY NITROGEN AND FOOD INTAKE

Group	Treatment	Ave. food intake		Ave. mg. N/100 gm. body weight/24 hrs.					
		Total gm.	Per gm. gain body wt. gm.	Before treatment	Treatment period in days				
					5	10	5	10	5	10
1	No vitamin B_{12}	486.0	8.04	141.80* ±22.0	188.75 ±26.1	203.38 ±1.37	181.75 ±8.7	186.75 ±21.9	181.5 ±29.6	176.52 ±33.9
2	Vitamin B_{12}	730.8	5.06	106.00* ±12.4	113.87 ±13.9	109.0 ±6.5	75.04 ±18.9	84.37 ±3.0	87.77 ±19.3	97.37 ±2.8
					Cortisone					
					1 mg.		2 mg.		4 mg.	
3	Cortisone	428.8	--	120.54* ±16.1	179.50 ±23.7	209.25 ±9.2	236.09 ±14.3	232.0 ±35.3	214.37 ±26.0	337.25 36.7
4	Cortisone + Vitamin B_{12}	535.8	11.80	149.24* ±10.9	127.12 ±21.0	96.37 ±1.8	90.68 ±0.1	72.5 ±3.5	164.87 ±10.5	153.25 ±23.3

*Average of 3 figures taken at 10-day intervals



gains. A partial explanation for this phenomenon will be provided in subsequent experiments.

Experiment II. Prevention by Vitamin B_{12} of Protein Catabolic Action of Cortisone

Purpose

In this experiment an attempt was made to confirm the results of Experiment I and in addition to determine whether vitamin B_{12} could prevent the increase in urinary nitrogen losses produced by injecting large doses of cortisone under limited food intake, as it did in the rats which were fed *ad libitum*.

Methods

Fifty rats were used in this experiment. The procedure was essentially the same as in Experiment I except for one additional group of rats. After being on the vitamin B_{12}-deficient diet for 20 days, the animals were divided into five uniform groups and were treated as follows for 30 days:

Group 1. No vitamin B_{12}

2. Vitamin B_{12} -- 200 mcg./kilogram of diet

3. Cortisone

4. Cortisone + Vitamin B_{12}

5. Cortisone + Vitamin B_{12}, but pair-fed to Group 3

Groups 3, 4 and 5 were treated with 1 mg. of cortisone daily for the first 10 days; 2 mg. daily for the second 10 days and 4 mg. daily for the third 10 days. Urinary nitrogen was measured every week during the depletion period and every

five days during the treatment period. Body weight and food consumption were measured every two days.

Results

The results are shown in Fig. 2. The first four groups followed essentially the same pattern as in the first experiment. The rats averaged 58.3 grams at the beginning of the depletion period and 94.9 grams at the end of the depletion period. Group 1, which continued to receive no vitamin B_{12} showed a final average body weight gain of 45 grams. Group 2 whose ration was supplemented with 200 mcg. of vitamin B_{12} per kilogram of diet, showed an average weight gain of 97.5 grams per rat. Cortisone (Group 3) caused a marked suppression of body growth. When vitamin B_{12} was added to the ration of the cortisone-injected rats (Group 4) it partially prevented the growth inhibition. These rats gained an average of 50 grams less than the vitamin B_{12}-supplemented rats in Group 2. When food intake was limited in Group 5 to that consumed by Group 3, vitamin B_{12} did not at all prevent the growth inhibition induced by cortisone.

Food intake and efficiency of utilization for body growth were greatest in the rats treated only with vitamin B_{12} (Group 2) and least in rats given cortisone without vitamin B_{12} (Group 3). Food intake was increased when vitamin B_{12} was given to the cortisone-treated rats (Group 4) but efficiency of food utilization was well below that of either Group 1 or 2.

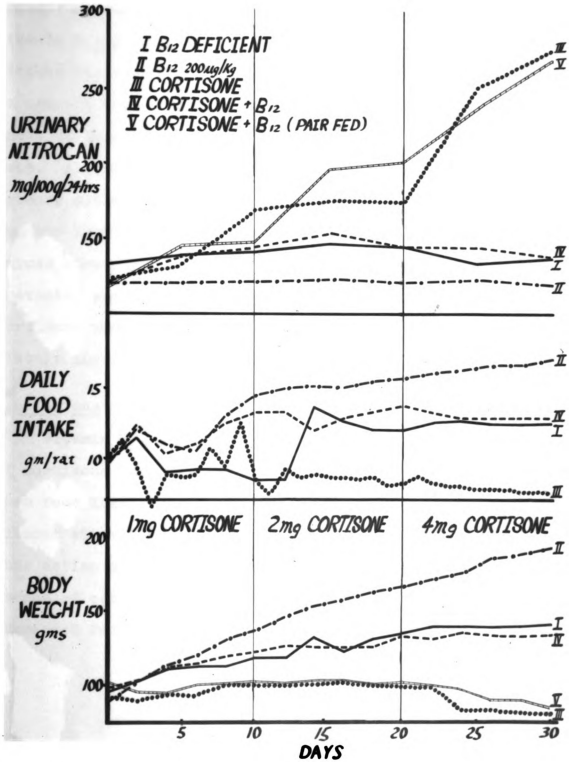


Fig. 2 Effects of vitamin B12 and cortisone on urinary nitrogen and food intake.

Table II shows that the average daily excretion of urinary nitrogen per 100 grams of body weight during the vitamin B_{12}-deficient period was not altered after the vitamin was added to the diet in Group 2. Nitrogen values in Group 1 were slightly higher than those of Group 2, although these differences were not as marked as in the first experiment. All levels of cortisone significantly increased urinary nitrogen losses in the vitamin B_{12}-deficient rats and the largest dose of hormone doubled the initial nitrogen values (Group 3). The addition of vitamin B_{12} to the ration prevented any increase in urinary nitrogen excretion by cortisone under ad libitum feeding (Group 4) but was completely ineffective when food intake was limited (Group 5).

Conclusions

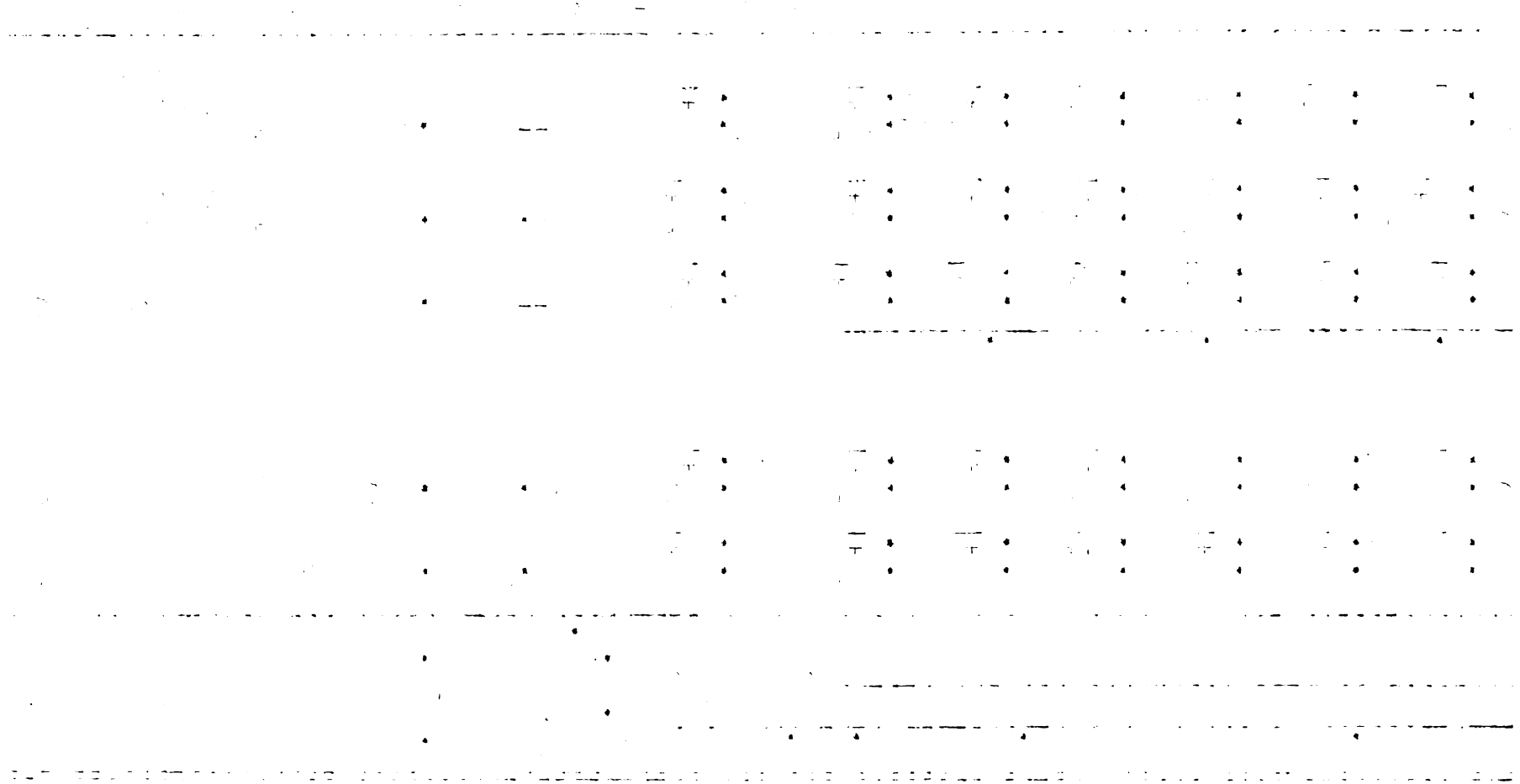
Vitamin B_{12} prevented the protein catabolic actions of cortisone under ad libitum feeding, but was ineffective when food intake was reduced to that of rats given cortisone without vitamin B_{12}. As explained in the first experiment, this action of vitamin B_{12} can be attributed to the greater amount of carbohydrate made available as a result of the increased food intake.

TABLE II

EFFECTS OF VITAMIN B_{12} AND CORTISONE ON URINARY NITROGEN AND FOOD INTAKE

Group	Treatment	Ave. food intake		Ave. mg. N/100 gm. body weight/24 hrs.						
		Total gm.	Per gm. gain body wt. gm.	Before treatment	Treatment period in days					
					5	10	5	10	5	10
1	No vitamin B_{12}	327.6	7.47	132.56* ± 5.52	139.51 ±4.68	141.92 ±4.87	146.87 ±12.5	144.01 ±2.02	133.87 ±9.70	136.16 ±8.50
2	Vitamin B_{12}	386.0	3.71	120.01* ±5.45	120.71 ±2.50	121.28 ±2.94	123.58 ±2.75	120.96 ±8.46	122.72 ±2.34	119.23 ±1.20
					Cortisone					
					1 mg.		2 mg.		4mg.	
3	Cortisone	251.9	--	123.34* ±4.39	132.68 ±19.43	169.42 ±30.71	175.69 ±37.38	169.85 ±22.19	251.43 ±4.65	276.78 ±2.64
4	Cortisone + Vitamin B_{12}	357.0	9.92	120.64* ±10.77	137.64 ±7.17	144.44 ±6.14	154.09 ±6.51	144.34 ±9.15	144.81 ±2.03	137.63 ±16.8
5	Cortisone + Vitamin B_{12} (pair fed)	251.9	--	118.14* ±8.00	145.34 ±0.18	148.46 ±0.10	192.24 ±11.45	201.59 ±6.00	238.74 ±10.5	270.70 ±0.47

*Average of 3 figures taken at 7-day intervals



Experiment III. Effects of Cortisone, Vitamin B_{12}, Insulin and Alloxan-diabetes on Blood Glucose and Urinary Glucose and Nitrogen

Purpose

This experiment was designed to provide information on the following:

1. Through what means does cortisone partially prevent vitamin B_{12} from exerting its full effects on efficiency of food utilization and body growth?
2. Does insulin require vitamin B_{12} for its action?
3. Does vitamin B_{12} require the presence of insulin for its action on food intake and body growth?

Methods

Sixty weanling male rats were fed the vitamin B_{12}-deficient stock diet for 60 days, when their body weights reached an average of approximately 160.0 grams each. They were then divided into six groups of 10 each and were treated as follows for 30 days:

Group 1. No vitamin B_{12} ✓
2. Vitamin B_{12} -- 200 mcg./kilogram of diet ✓
3. Alloxan -- 17.5 mg./100 grams
4. Alloxan + Vitamin B_{12}
5. Cortisone -- 4 mg./day/rat
6. Cortisone + Vitamin B_{12}

Food intake and body weight were measured every two days. In accordance with standard procedure, all rats which were to

receive alloxan were starved for 72 hours. In order to maintain the same body weight in all rats, the rats which were not treated with alloxan were also starved for 72 hours. At the end of this period, Groups 3 and 4 were injected with alloxan monohydrate. At the end of five days, hyperglycemia was established.

Blood glucose was determined on the 5th, 10th, 20th and 30th days by the micro-method of Folin and Malmros (Hawk et al. 1951). This procedure was used because less blood was required and lower blood levels could be determined than with the Hartman, Shaeffer and Somogyi micro-method.

After each initial collection of blood, 0.5 units of insulin were injected into the rats of Groups 1 and 2 and 2.0 units were injected into the rats of the other four groups. Blood samples were collected four hours later.* The insulin dosage was less in Groups 1 and 2, because in preliminary experiments, it was found that 2.0 units of insulin injected into vitamin B_{12}-deficient and -adequate control rats produced a severe and often fatal hypoglycemia. Urinary glucose was determined by the method of Hawkins and Van Slyke (Hawk et al. 1951) and urinary nitrogen by the micro-Kjeldahl procedure previously described. The rats were starved 12 hours prior to insulin injections and for four hours subsequently.

*Sugar determinations were made four hours after insulin injection because it was found that blood glucose fell to the lowest point at this time. See appendix for experiment in which this was determined.

Results

1. Effects on body weight and food intake (Table III and Fig. 3).

The rats in Group 1 which were fed the vitamin B_{12}-deficient diet gained an average of 43 grams each as compared to the vitamin-fed rats in Group 2 which gained an average of 88 grams each. Alloxan slowed body growth in the vitamin-deficient rats (Group 3), and the average total gain was only about 20 grams. In the vitamin B_{12}-adequate animals given alloxan (Group 4), the average total gain was approximately 77 grams. Cortisone decreased body growth more in the vitamin B_{12}-deficient animals (Group 5) than in the vitamin B_{12}-adequate rats (Group 6). The latter is in agreement with the results of the previous experiments.

Food intake was reduced in all groups which did not receive vitamin B_{12}, and was increased when the vitamin was added to the diet. It will be noted that in the alloxan-diabetic rats fed vitamin B_{12} (Group 4), food intake, body weight and efficiency of food utilization were about the same as in the normal rats fed vitamin B_{12} (Group 2). As in the two previous experiments, it can be seen that cortisone reduced the ability of vitamin B_{12} to convert food into body weight gains (Group 6).

2. Effects on blood and urinary glucose and urinary nitrogen (Table IV and Figs. 3 and 4).

The average blood glucose in the vitamin-deficient rats

TABLE III

EFFECTS OF VITAMIN B_{12}, ALLOXAN, AND CORTISONE ON BODY WEIGHT AND FOOD INTAKE

Group	Treatment	Body weight gm.		Food intake gm.	
		Initial	Final	Total	Efficiency
1	No vitamin B_{12}	176.7	219.8	302.6	7.02
2	Vitamin B_{12} 200 mcg./kilogram	169.8	257.1	402.8	4.60
3	Alloxan 17.5 mg/100 gm.	170.7	191.0	285.2	14.04
4	Alloxan + Vitamin B_{12}	169.6	246.1	398.2	5.20
5	Cortisone 4 mg./day	170.8	148.3	257.2	--
6	Cortisone + Vitamin B_{12}	169.9	215.3	345.3	7.60

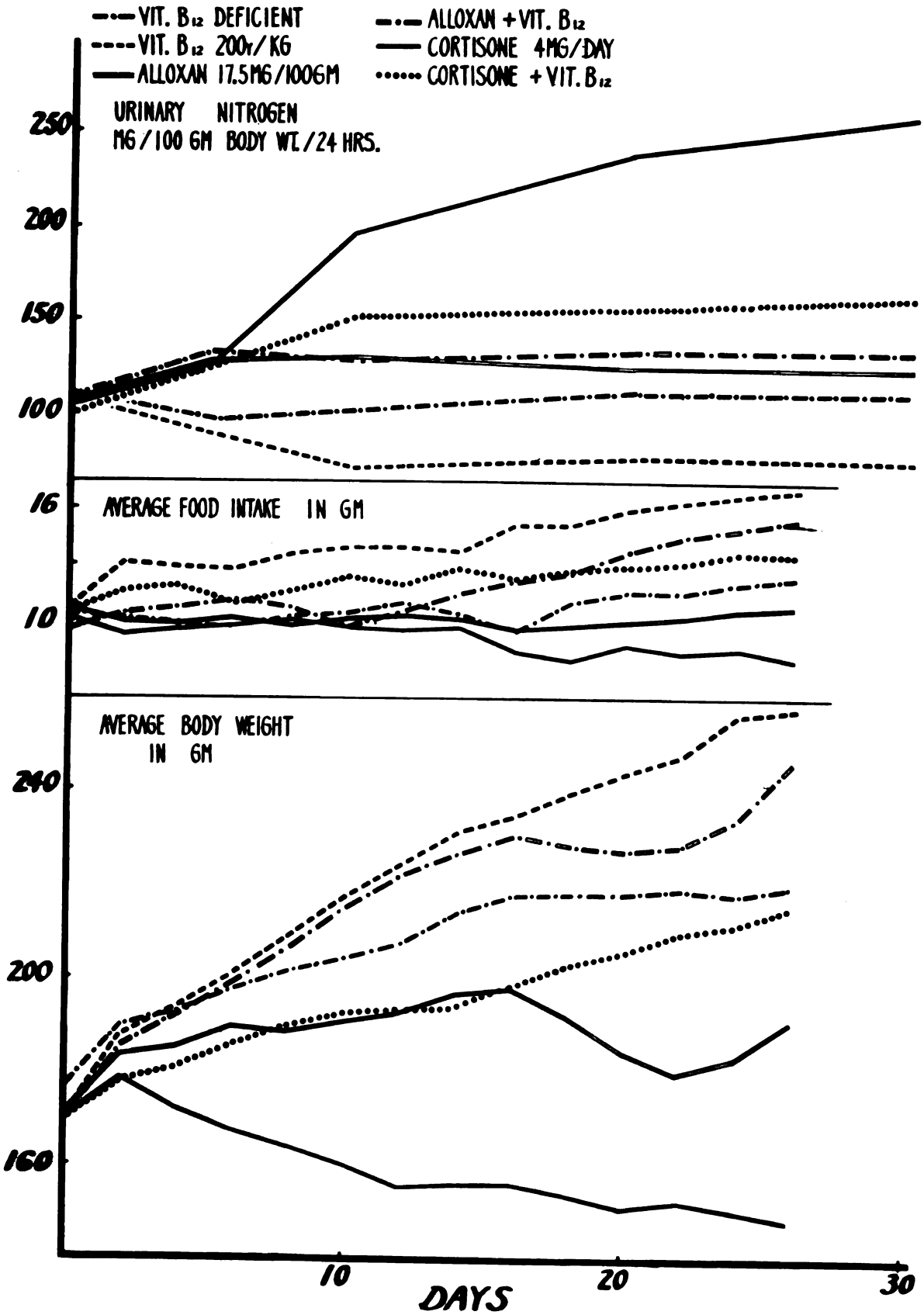


Fig. 3 Effects of alloxan, cortisone and vitamin B_{12} on body weight, food intake and urinary nitrogen.

TABLE IV

EFFECTS OF ALLOXAN, CORTISONE, AND VITAMIN B_{12} on BLOOD AND URINARY GLUCOSE AND URINARY NITROGEN

B.G. = Blood glucose = mg. %
U.G. = Urinary glucose = gm./100 gm./24 hrs.
U.N. = Urinary nitrogen = mg./100 gm./24 hrs.

Group	Treatment	Initial	5 days	10 days	20 days	30 days
1	No vitamin B_{12}					
	B.G.	84.4±4.7	84.7±4.3	84.8±5.6	85.1±4.8	86.2±5.1
	U.G.	--	--	--	--	--
	U.N.	100.50	97.36	102.84	118.12	117.04
2	Vitamin B_{12}					
	B.G.	86.0±5.2	95.9±5.1	113.9±4.0	116.9±3.9	118.1±4.6
	U.G.	--	--	--	--	--
	U.N.	108.2	90.9	72.9	77.8	75.9
3	Alloxan					
	B.G.	83.8±5.1	169.4±10.5	183.5±7.9	188.9±6.8	188.0±6.4
	U.G.		1.649	1.926	1.862	1.540
	U.N.	107.98	128.85	131.71	126.02	130.57
4	Alloxan + Vitamin B_{12}					
	B.G.	82.9±4.4	257.5±4.9	273.9±8.9	277.2±8.1	287.5±12.5
	U.G.		2.454	2.872	2.693	2.502
	U.N.	110.19	134.29	130.38	134.88	91.64
5	Cortisone					
	B.G.	82.1±7.0	185.5±8.9	269.4±8.8	351.2±15.5	380.2±24.3
	U.G.		1.432	2.872	2.976	3.725
	U.N.	106.01	127.72	197.48	239.12	258.88
6	Cortisone + Vitamin B_{12}					
	B.G.	85.4±4.4	157.3±3.6	202.7±10.1	210.3±6.5	213.9±14.6
	U.G.		1.242	1.827	1.942	2.064
	U.N.	100.69	115.96	152.42	157.37	162.82

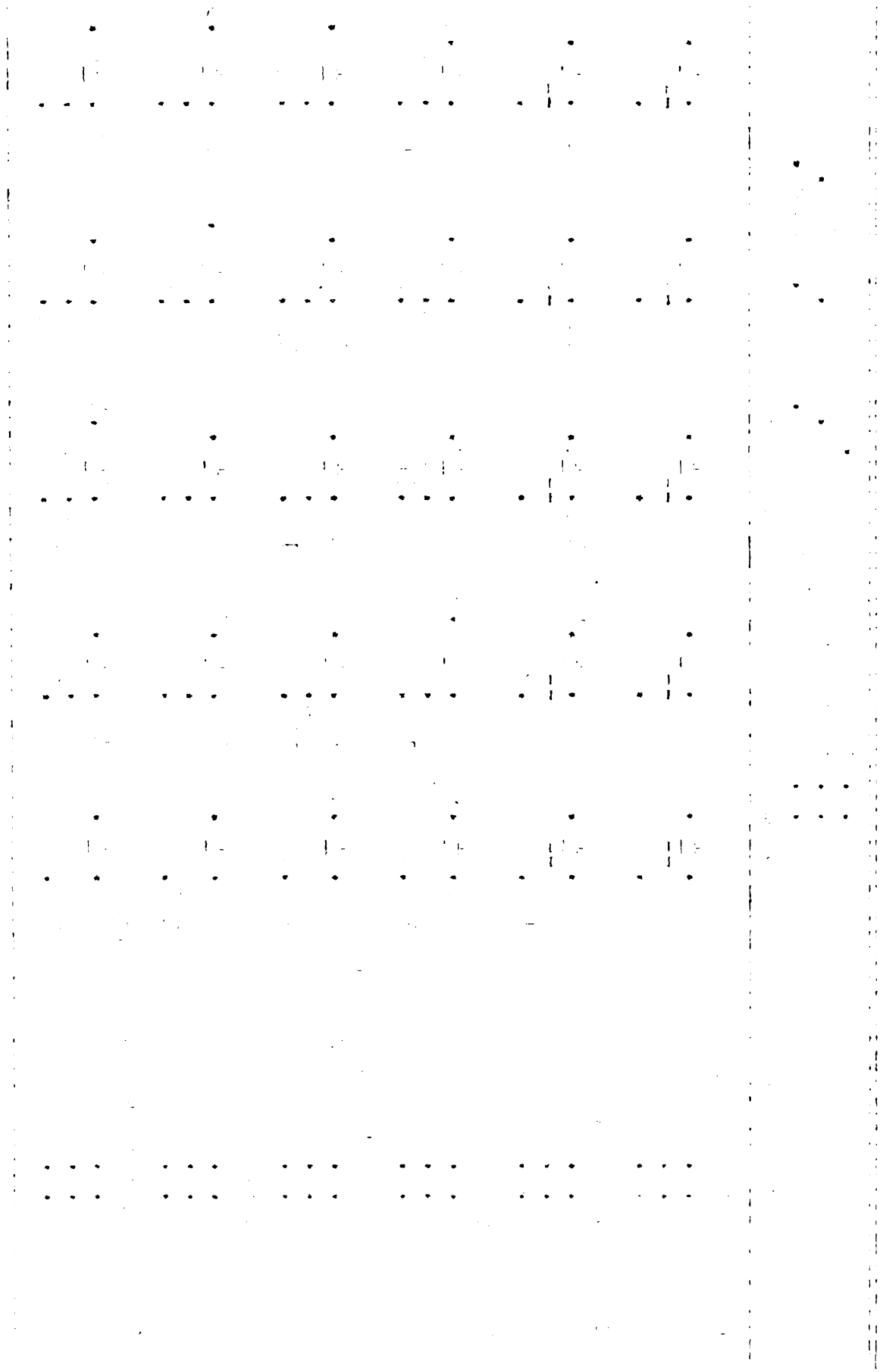


Fig. 4 Effect of insulin on blood and urinary glucose after pretreatment with alloxan, cortisone and vitamin B_{12}

(Group 1) was somewhat lower than in the vitamin-adequate rats in Group 2 throughout the experiment. The former averaged approximately 84.5 ± 5.0 mg. percent and the latter about 115 ± 4.0 mg. percent. Alloxan did not induce as much hyperglycemia and glucosuria in the vitamin B_{12}-deficient rats (Group 3) as in the vitamin B_{12}-adequate rats (Group 4). The blood glucose in the former increased from 82.8 to 169.4 mg. percent on the 5th day, to 183.5 mg. percent on the 10th day, to 188.9 mg. percent on the 20th day and to 188.3 mg. percent on the 30th day. In the latter rats, the blood glucose reached 257.5 mg. percent on the 5th day, 273.9 mg. percent on the 10th day, 277.2 mg. percent on the 20th day and 287.5 mg. percent on the 30th day. Cortisone increased the blood glucose level to a high of 380.2 mg. percent in the vitamin B_{12}-deficient rats (Group 5), while in the vitamin B_{12}-adequate rats cortisone increased blood glucose to only 213.9 mg. percent at the end of 30 days of treatment. The urinary glucose values followed those in the blood. Group 5 had higher blood glucose level and higher urinary glucose level while Group 6 had lower blood glucose level and less glucose in the urine.

In general, the nitrogen excretion pattern in the urine followed the results obtained in the previous experiments. It should be noted that in the vitamin B_{12}-deficient rats (Group 5) cortisone increased blood glucose and urinary glucose and nitrogen to a greater extent than in the vitamin-

adequate rats (Group 6). Alloxan increased urinary nitrogen excretion about the same under both dietary treatments.

3. Effects of insulin (Table V and Fig. 4).

Comparisons of Groups 1 and 2 after insulin injection show that the vitamin B_{12}-deficient animals were less reactive to this hormone than the vitamin-adequate rats. Blood glucose fell only about 33 to 37 percent in the former as compared to 53 to 54 percent in the latter. In the alloxan diabetic rats insulin reduced blood glucose only one-half as much in the vitamin B_{12}-deficient (Group 3) as in the vitamin-adequate rats (Group 4); however, the percentage reduction was about the same in both groups. The vitamin B_{12}-deficient rats treated with cortisone (Group 5) showed the greatest resistance to insulin of any group, with only an average reduction in the three trials of about 24 percent in blood glucose, while the vitamin B_{12}-adequate animals treated with cortisone (Group 6) showed an average reduction of about 66 percent.

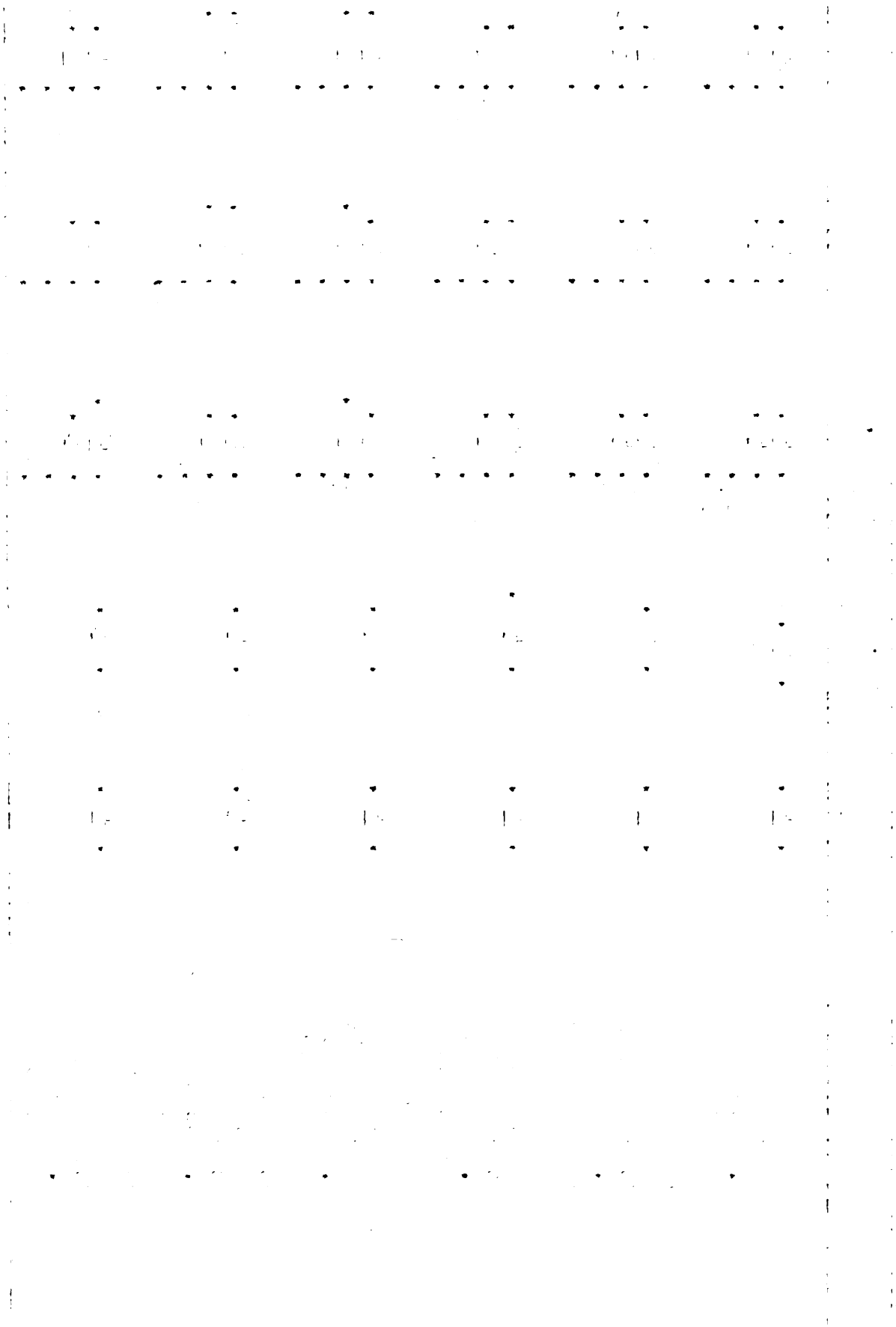
Conclusions

1. Vitamin B_{12} increased body growth by increasing appetite and increasing efficiency of food utilization. Alloxan slowed body growth in the vitamin B_{12}-deficient rats, but not in the vitamin-adequate animals. This was accompanied by increased food intake and food utilization which was practically equal to that of the normal vitamin B_{12}-treated rats. This demonstrates that insulin is not

TABLE V

EFFECTS OF INSULIN ON BLOOD GLUCOSE AFTER PRETREATMENT WITH ALLOXAN, CORTISONE AND VITAMIN B_{12}

Group	Treatment	Blood glucose mg. % Initial	5 days	10 days	20 days	30 days
1	No vitamin B_{12}					
	Before insulin	84.4±4.7	84.7±4.6	84.8±5.6	85.1±4.8	86.2±2.1
	After insulin			53.6±3.4	57.0±2.4	56.5±4.4
	Mg. decrease			31.22	28.1	39.7
	Percent decrease			36.8	33.0	34.4
2	Vitamin B_{12}					
	Before insulin	86.0±5.2	95.9±5.1	113.9±4.0	116.9±3.9	118.1±9.5
	After insulin			53.0±3.1	54.7±2.6	55.2±3.3
	Mg. decrease			60.9	62.2	63.9
	Percent decrease			53.5	53.2	53.2
3	Alloxan					
	Before insulin	83.8±5.1	169.4±10.5	183.5±7.9	188.9±6.8	188.0±1.5
	After insulin			56.1±4.3	50.0±4.2	53.4±3.7
	Mg. decrease			127.3	138.9	134.6
	Percent decrease			69.4	73.5	71.7
4	Alloxan + Vitamin B_{12}					
	Before insulin	82.9±4.4	257.5±4.9	273.9±8.9	277.2±8.1	287.5±11.0
	After insulin			101.4±12.3	98.7±11.8	103.9±11.8
	Mg. decrease			172.5	178.5	184.5
	Percent decrease			62.9	64.4	64.2
5	Cortisone					
	Before insulin	82.1±7.0	185.5±8.9	269.4±8.8	351.2±15.5	380.2±16.6
	After insulin			193.0±9.6	275.8±13.3	294.0±10.8
	Mg. decrease			76.3	75.3	86.2
	Percent decrease			28.4	21.5	22.7
6	Cortisone + Vitamin B_{12}					
	Before insulin	85.4±4.4	157.3±3.6	202.7±10.1	210.3±6.5	213.9±4.3
	After insulin			54.8±3.8	75.2±3.6	75.2±3.1
	Mg. decrease			147.9	135.1	138.7
	Percent decrease			72.9	64.2	64.9



essential for the growth promoting action of vitamin B_{12}. Cortisone retarded body growth and food intake in the vitamin B_{12}-deficient rats to a much greater extent than in the vitamin-adequate rats. It will be noted again that cortisone partially interfered with the action of vitamin B_{12} on food efficiency and body growth.

2. Vitamin B_{12} maintained blood glucose levels somewhat higher than in the vitamin B_{12}-deficient animals. Alloxan diabetes raised blood glucose to a much higher level in the vitamin B_{12}-adequate than in the vitamin-deficient rats. This can be attributed to the ability of vitamin B_{12} to increase food intake. The urinary glucose was also higher in the vitamin-treated animals. It is of interest, however, that these alloxan-diabetic animals fed vitamin B_{12} weighed more and used their food almost as efficiently for making gains in weight as the normal vitamin B_{12}-fed rats. This indicates that the ability to convert food into body weight is not seriously interfered with in the absence of insulin if a sufficient amount of vitamin B_{12} is available. It will be noted that in the absence of insulin, vitamin B_{12} did not alter urinary nitrogen excretion. The greatest hyperglycemia and glucosuria was produced by cortisone in the vitamin B_{12}-deficient rats, apparently by interfering with insulin action (Ingle *et al.* 1946) and by increasing gluconeogenesis. Vitamin B_{12} partially counteracted the catabolic action of cortisone by reducing gluconeogenesis from protein. Thus

both urinary nitrogen and blood and urinary glucose were reduced by treatment with vitamin B_{12}. This is believed to further explain how large doses of vitamin B_{12} can partially counteract the protein-catabolic effects of cortisone.

3. Insulin was generally less effective in reducing blood glucose in the vitamin B_{12}-deficient groups than in the vitamin-adequate groups with possible exception in the alloxan-diabetic rats. This indicates that the vitamin is essential for the full and maximum effect of insulin to be manifested, and in this respect it is similar to some other B-vitamins (Samuels, 1948). It is probable that the ability of insulin to favor the transformation of glucose into fat depends in part on the presence of vitamin B_{12}. By far the greatest resistance to insulin was encountered in the cortisone-treated rats on the vitamin B_{12}-deficient diet (Group 5). This apparently was due to: 2) an interference by cortisone with insulin action, and is in agreement with similar observations by Ingle *et al.* (1945) and b) to the decreased effectiveness of insulin in the absence of vitamin B_{12}.

4. Cortisone partially interfered with the favorable action of vitamin B_{12} on efficiency of food utilization and body growth, apparently by inducing hyperglycemia and glucosuria and thereby leaving less carbohydrate available for conversion into body weight gains. The hyperglycemia and glucosuria are undoubtedly related in part to cortisone-induced insulin resistance. In normal rats fed vitamin B_{12},

there was no loss of sugar in the urine. It would have been interesting to see whether injections of insulin into cortisone-treated, vitamin B_{12}-fed rats would not have further prevented decreases in body growth.

Experiments IV and V. Glucose Utilization in Normal, Alloxan-diabetic and Cortisone-treated Rats as Influenced by Vitamin B_{12}

Purpose

Chow et al. (1954) demonstrated that glucose tolerance was lower in vitamin B_{12}-deficient than in vitamin B_{12}-adequate rats. In these experiments, it was intended to see whether these results could be confirmed and also to determine the effects of alloxan-diabetes and cortisone on glucose utilization in vitamin B_{12}-deficient and -adequate rats.

Methods

The 60 rats from Experiment III were used in this study. At tne end of 33 days of treatment they were starved for 12 hours, blood samples were taken from each rat, and initial glucose values were determined. A total of 750 mg. of glucose in 5 ml. of physiological saline was injected intraperitoneally into each rat. Blood samples were taken one and two hours later for glucose determinations. Twenty-four hours later a similar dose of glucose was injected into each rat and urine was collected for six hours for glucose assays. The preceding was repeated after an interval of six days.

Results of Experiment IV

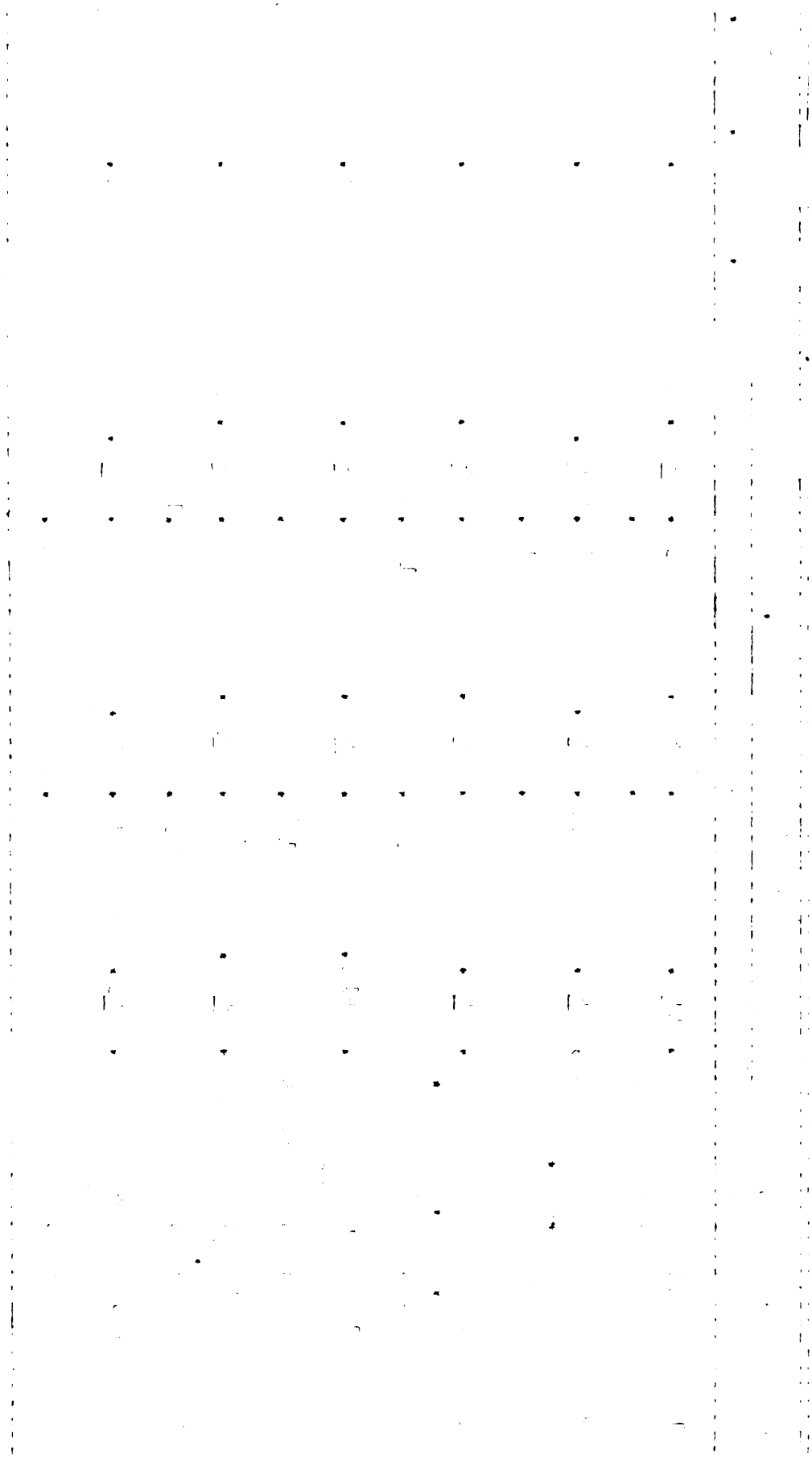
The results are shown in Table VI and Fig. 5. Apparently the vitamin B_{12}-deficient rats (Group 1) had little ability to utilize the injected glucose, since their blood

TABLE VI

TABLE VI

EFFECTS OF VITAMIN B_{12}, ALLOXAN AND CORTISONE ON GLUCOSE TOLERANCE TEST

Group	Treatment	Blood glucose mg. %			Urinary glucose
		Initial	1 hour	2 hours	gm./100 gm./6 hrs.
1	No vitamin B_{12}	85.01±1.48	640.88±22.26	430.62±26.43	0.48
	Percent increase		653.9	406.5	
2	Vitamin B_{12} 200 mcg./kg.	123.70±3.09	233.09±7.12	136.10±2.38	0.098
	Percent increase		89.4	10.0	
3	Alloxan 17.5 mg./100 gm.	189.51±6.12	748.02±15.50	478.04±22.6	0.97
	Percent increase		301.1	152.3	
4	Alloxan + Vitamin B_{12}	286.13±13.0	748.62±22.83	489.72±29.32	1.25
	Percent increase		159.9	71.2	
5	Cortisone 4 mg./day	355.18±15.0	868.09±39.0	676.02±38.83	1.67
	Percent increase		137.7	85.1	
6	Cortisone + Vitamin B_{12}	181.70±3.16	313.93±9.50	235.37±9.80	0.76
	Percent increase		72.8	29.5	



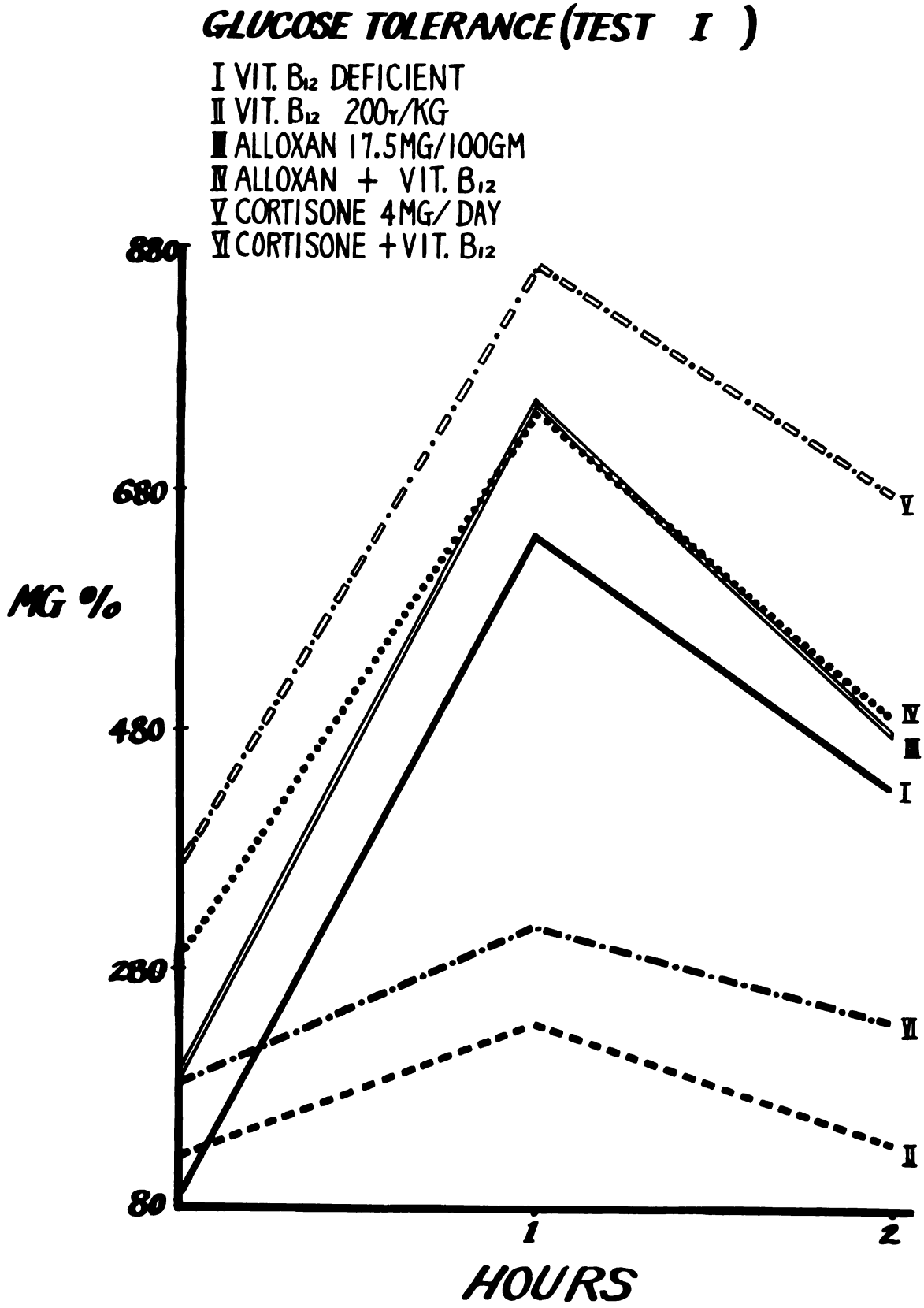


Fig. 5 Effects of vitamin B12, alloxan and cortisone on glucose tolerance test.

glucose reached levels almost three times as high as in the vitamin-adequate animals (Group 2). Also the former excreted about four times as much glucose in the urine as the latter. Both the vitamin B_{12}-deficient and -adequate alloxan-diabetic rats (Groups 3 and 4) showed the same elevated blood glucose levels, but the percentage increase was much greater in the vitamin-deficient rats. There was somewhat more glucose excreted in the urine of the latter rats. Cortisone elevated glucose to a much higher level in the vitamin B_{12}-deficient rats (Group 5) than in the vitamin-adequate rats (Group 6), despite the fact that blood glucose was initially much higher in the former. There was also about twice as much glucose excreted by the vitamin B_{12}-deficient as by the vitamin-adequate rats treated with cortisone.

Results of Experiment V

It can be seen in Table VII and Fig. 6 that the results are essentially the same as in Experiment IV.

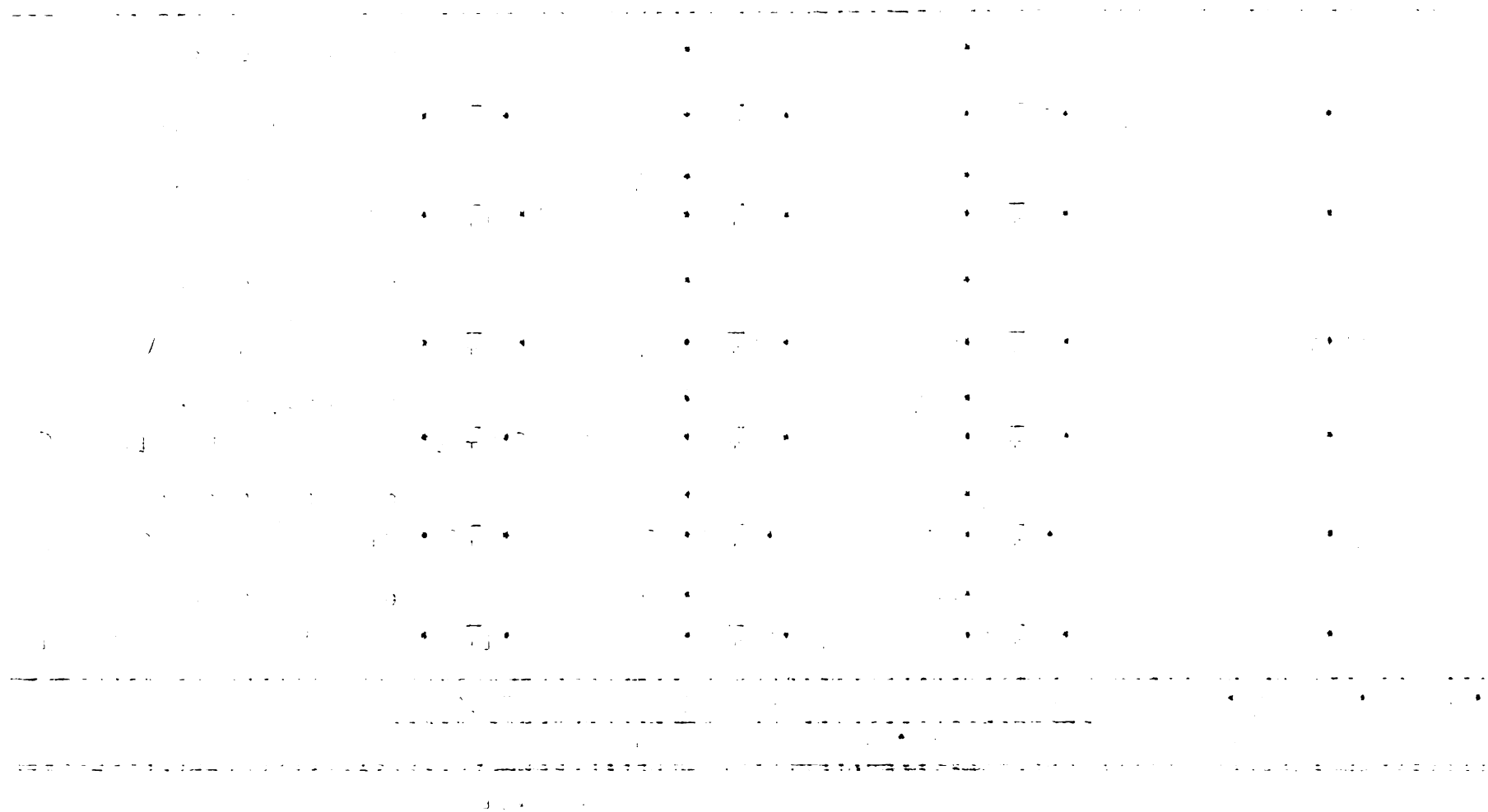
Conclusions

These data confirm the report of Chow et al. (1954) that a vitamin B_{12}-deficiency interferes with the utilization of glucose by rats. This was also shown to be true in alloxan-diabetic and cortisone-treated rats. In general, these results are believed to suggest that vitamin B_{12} may increase glucose utilization independently of insulin. Large amounts of cortisone can partially interfere with

TABLE VII

EFFECTS OF VITAMIN B_{12}, ALLOXAN AND CORTISONE ON GLUCOSE TOLERANCE TEST

Group	Treatment	Blood glucose mg. %			Urinary glucose
		Initial	1 hour	2 hours	gm./100 gm./6 hrs.
1	No vitamin B_{12}	85.22±1.04	639.72±23.87	417.16±22.56	0.52
	Percent increase		650.7	389.5	
2	Vitamin B_{12}	124.83±1.50	233.33±5.10	136.93±2.29	0.079
	Percent increase		86.9	9.7	
3	Alloxan	187.38±5.35	748.82±14.45	480.71±20.28	0.98
	Percent increase		299.6	156.5	
4	Alloxan + Vitamin B_{12}	287.29±13.43	755.11±19.74	484.25±23.74	1.30
	Percent increase		162.8	68.6	
5	Cortisone	367.06±13.49	884.40±12.42	685.82±13.35	1.74
	Percent increase		140.9	86.8	
6	Cortisone + Vitamin B_{12}	184.60±3.08	321.93±10.51	230.75±13.45	0.68
	Percent increase		74.4	25.0	



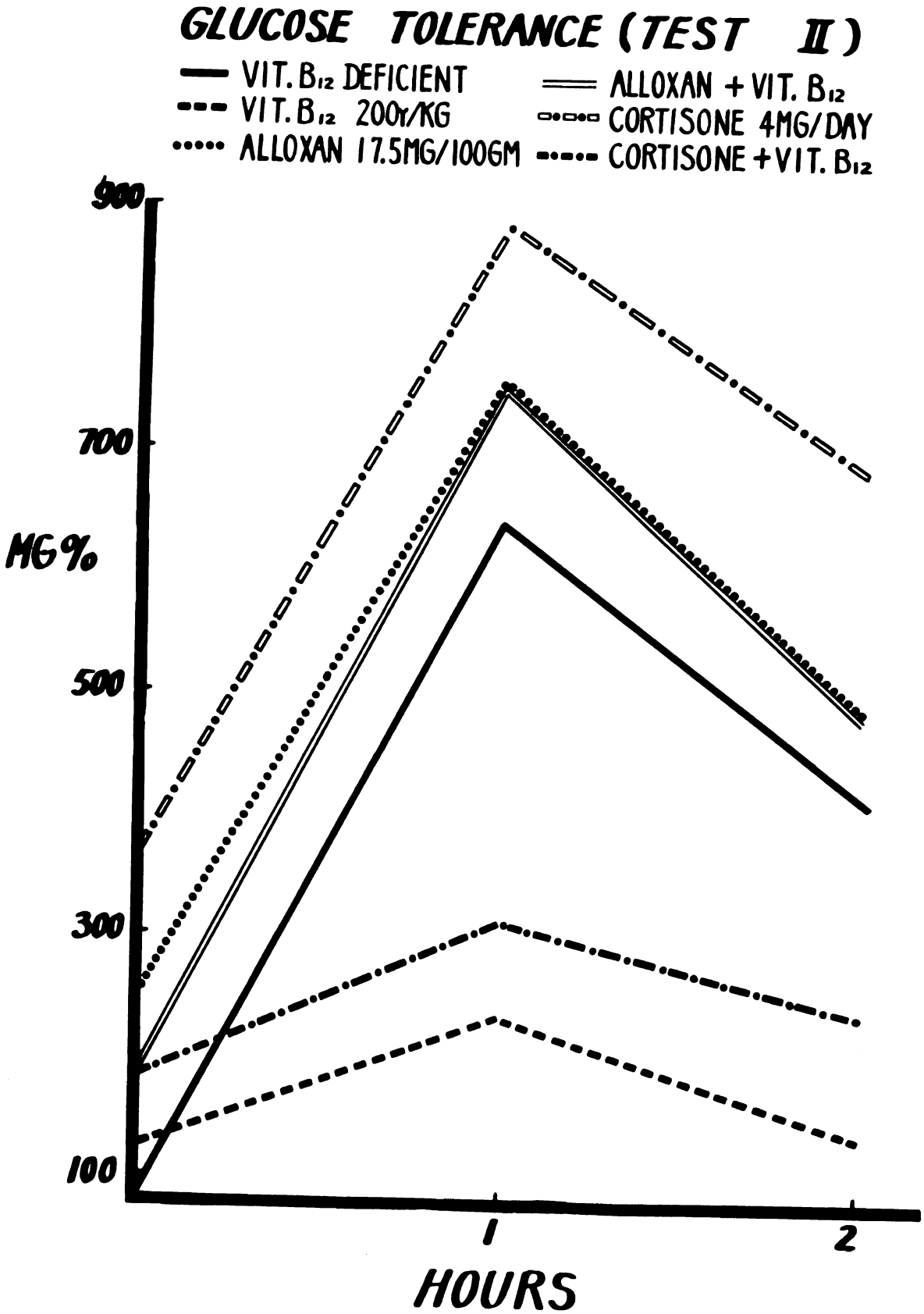
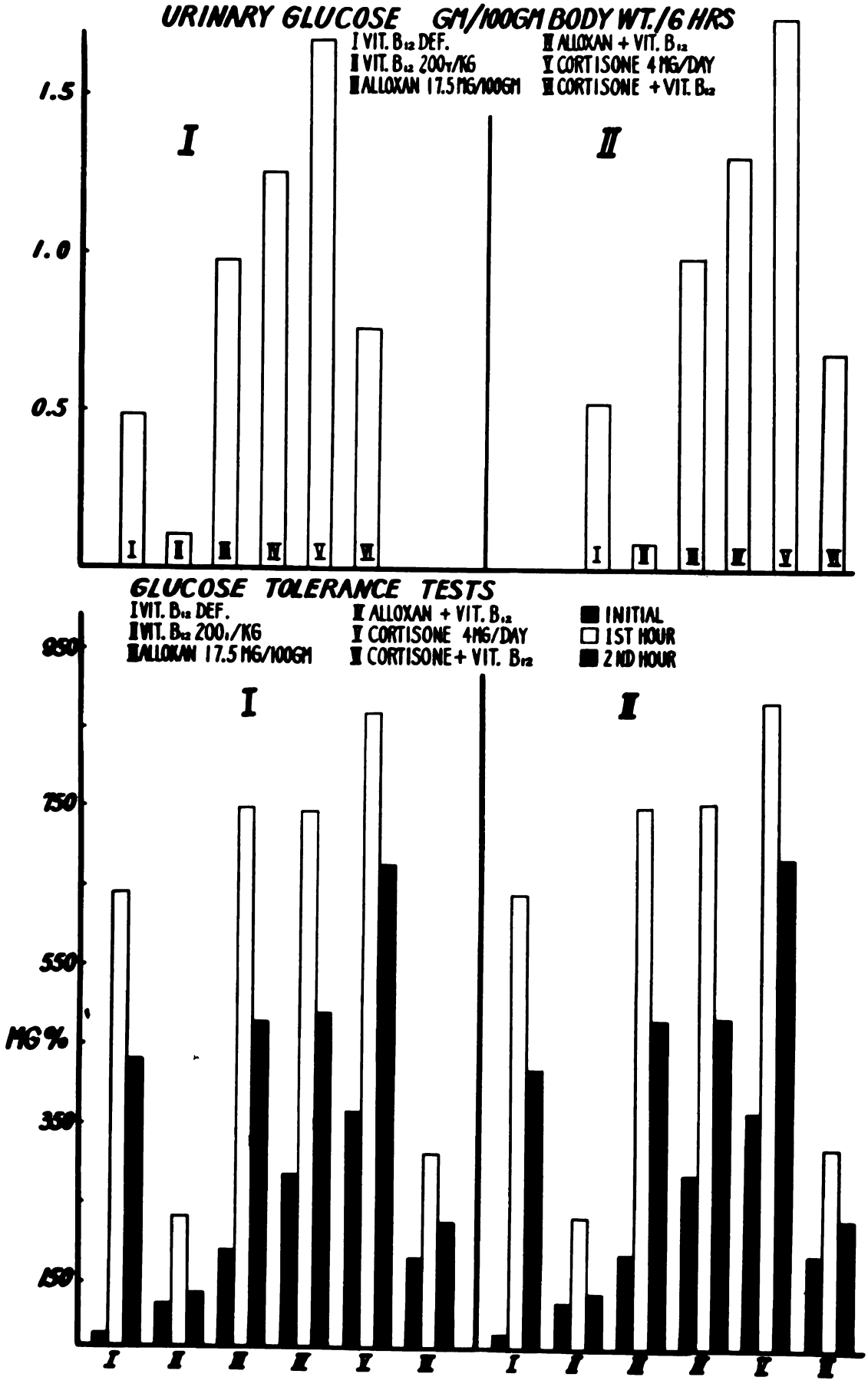


Fig. 6 Effects of vitamin B12, alloxan and cortisone on glucose tolerance test.



Effects of vitamin B12, alloxan and cortisone on glucose tolerance test.

this action of the vitamin. The actions of the pancreas and cortisone on glucose metabolism do not appear entirely independent of vitamin B_{12}. Thus a vitamin B_{12} deficiency enhances the ability of cortisone to increase gluconeogenesis from protein and hampers insulin in increasing glucose utilization. On the other hand, an excess of vitamin B_{12} decreases the action of cortisone on gluconeogenesis and enhances insulin function. Since adrenal cortical action on carbohydrate formation is unopposed in diabetic animals, it is suggested that large doses of vitamin B_{12} may partially substitute for insulin in maintaining carbohydrate utilization and body growth. The latter is purely conjectural and may have to be demonstrated.

Experiment VI. Effects of Cortisone on Distribution of Vitamin B_{12} in Blood, Liver and Urine

Purpose

Chow et al. (1951) determined the distribution of radioactive vitamin B_{12} in normal and vitamin B_{12}-deficient rats, and found that the former retained less of this vitamin than the latter. Wahlstrom and Johnson (1951) stated that large doses of cortisone injected into baby pigs increased urinary excretion of vitamin B_{12}. It was desired in this experiment to study the effects of cortisone on the distribution of radioactive vitamin B_{12} in blood, liver and urine of vitamin B_{12}-deficient and -adequate rats.

Methods

At the end of Experiment II, five rats from each group were injected intraperitoneally with 5 mcc. of radioactive vitamin B_{12} (labeled with Co^{60}) and were placed in metabolism cages for collection of 24-hour urine specimens. At the end of this period, they were killed by decapitation, and 1 ml. of blood was collected from each rat. Whole liver weights were recorded and approximately 250 mg. samples from each rat were removed for counting radioactivity. Both blood and liver samples were ashed in a muffle furnace for two hours. Urine samples were prepared by drying 2 ml. of urine in a crucible cover. All samples were counted under a Geiger-Muller end window counter for ten minutes. Corrections

were made for background. The results were calculated as counts per second per ml. of blood, counts per second per 100 mg. of liver and counts per second per 24-hour urine specimen per 100 grams of body weight.

Results

In Table VIII it can be seen that vitamin B_{12}-deficient rats (Group 1) retained considerably more radioactive vitamin B_{12} in their tissues and excreted less than half as much in the urine as the vitamin B_{12}-adequate rats (Group 2). The cortisone-treated, vitamin B_{12}-deficient rats (Group 3) had approximately the same amount of radioactive vitamin B_{12} in the blood, but less was concentrated in the liver and about three times as much was lost in the urine as in Group 1. When cortisone was given to vitamin B_{12}-adequate rats (Group 4), the distribution of the radioactive vitamin was about the same as in Group 2, indicating that the hormone did not alter the retention of vitamin B_{12} in these rats. When food intake was limited (Group 5), cortisone slightly increased the excretion of vitamin B_{12} in the urine.

Conclusions

These results confirm the reports of Chow et al. (1951) and Wahlstrom and Johnson (1951) that more vitamin is retained in animals which are deficient in this vitamin, and that cortisone increases the excretion of vitamin B_{12} in vitamin B_{12}-deficient animals. Although there may be some questions

TABLE VIII

EFFECTS OF CORTISONE AND VITAMIN B_{12} ON DISTRIBUTION OF Co^{60}-VITAMIN B_{12} IN BLOOD, LIVER AND URINE

Group	Treatment	Distribution of Co^{60}-Vitamin B_{12}		
		Blood cps/ml.	Liver cps/mg.	Urine cps/100 gm. body weight/24 hrs.
1	No vitamin B_{12}	0.219	0.104	10.87
2	Vitamin B_{12} 200 ug./kilogram	0.149	0.078	24.69
3	Cortisone	0.214	0.083	35.66
4	Cortisone + Vitamin B_{12}	0.179	0.096	26.88
5	Cortisone + Vitamin B_{12} (pair-fed)	0.187	0.093	30.65

as to whether the activity measured was actually vitamin B_{12}, this can be assumed since Chow *et al.* (1951, 1953) showed that biological and radioactive vitamin B_{12} values in tissues and urine of rats were in close agreement. It should be recalled that the amount of vitamin B_{12} fed the rats in Groups 2, 4 and 5 was about ten times above normal requirements, and hence it is not surprising that cortisone failed to alter markedly the distribution or excretion of vitamin B_{12} in these animals.

Experiment VII. Excretion of Radioactive Vitamin B_{12} in the Urine following Injection of Cortisone at Different Levels

Purpose

This experiment was intended to provide further information on the effects of cortisone on urinary vitamin B_{12} losses. Specifically, it was desired to determine the effects of different levels of cortisone in vitamin B_{12}-deficient rats and in rats fed only normal vitamin B_{12} requirements.

Methods

Thirty young male rats were initially fed the vitamin B_{12}-deficient stock diet for 60 days and were then divided into six uniform groups of five each. Three groups were continued on the stock soybean meal diet and the other three groups were fed the same diet supplemented with vitamin B_{12} in amounts of 20 mcg. per kilogram of ration for 10 days. Twenty mcg. of vitamin B_{12} per kilogram of diet is believed to represent the normal requirement for growing rats (Stokstad et al. 1949; Zucker et al. 1950).

Beginning on the 11th day, the rats were treated for 20 days as follows:

Vitamin B_{12}-deficient groups:

Group 1. Controls

2. 2 mg. Cortisone daily

3. 4 mg. Cortisone daily.

Vitamin B_{12}-fed groups:

Group 4. Controls

5. 2 mg. Cortisone daily

6. 4 mg. Cortisone daily

Food intake and body weight were measured every two days. Urinary nitrogen was determined every five days. After 20 days of the above treatment, the rats were injected with 0.1 mcc. of radioactive vitamin B_{12} intraperitoneally. Twenty-four-hour urine specimens were collected and vitamin B_{12} activity was determined as in the previous experiment.

Results

Body weight, food intake, food utilization per gram of body weight and urinary nitrogen excretion are shown in Table IX and Fig. 7. On the whole, these results are quite similar to those reported in Experiments I and II. Therefore only the urinary vitamin B_{12} excretion values will be considered here.

In the vitamin B_{12}-deficient rats cortisone greatly increased the excretion of the radioactive vitamin. Two mg. of the hormone daily doubled the loss of the vitamin and 4 mg. daily tripled its loss in the urine. On the vitamin-adequate diet, only the 4 mg. level of cortisone increased the loss of vitamin B_{12} in the urine.

TABLE IX

EFFECTS OF DIFFERENT LEVELS OF CORTISONE ON EXCRETION OF RADIOACTIVE VITAMIN B_{12} IN URINE

Group	Treatment	Body weight gm.			Food intake gm.	
		Pre-treatment	Beginning treatment	Final	Total	Food efficiency
	Vitamin B_{12}-deficient					
1	Control	154.2	170.0	194.0	180.8	7.53
2	2 mg. cortisone daily	154.0	173.0	169.0	188.6	--
3	4 mg. cortisone daily	153.0	169.0	134.0	149.4	--
	Vitamin B_{12}-fed					
4	Control	154.0	193.0	246.0	238.8	4.99
5	2 mg. cortisone daily	153.4	191.0	206.0	231.0	--
6	4 mg. cortisone daily	154.1	191.6	180.8	212.1	--

TABLE IX Continued

Group	Treatment	Ave. mg. N/100 gm. body weight/24 hrs.			Co^{60}-vitamin B_{12} in urine cps/100 gm./24 hrs. 0.1 mcg. I.P.
		5 days	10 days	17 days	
	Vitamin B_{12}-deficient				
1	Control	121.1	97.6	99.6	0.5417
2	2 mg. cortisone daily	146.6	140.4	141.1	1.1760
3	4 mg. cortisone daily	152.2	194.7	222.1	1.8238
	Vitamin B_{12}-fed				
4	Control	92.5	88.5	99.1	0.8265
5	2 mg. cortisone daily	128.7	121.8	127.4	0.5949
6	4 mg. cortisone daily	147.8	130.2	137.4	1.2391

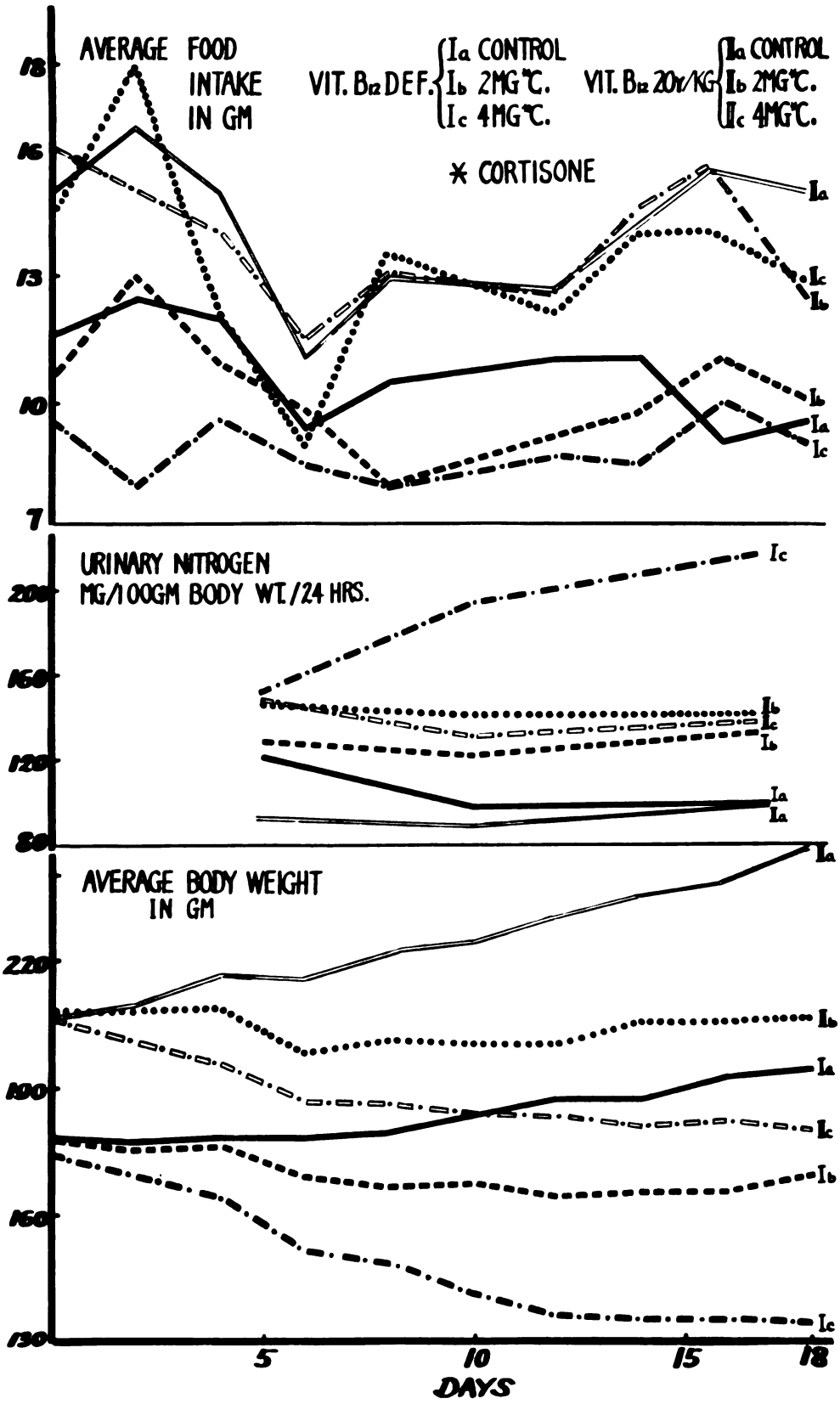


Fig. 7 Effects of different levels of cortisone on body weight, food intake and urinary nitrogen.

Conclusions

On the whole, these results confirm those in the previous experiment. In addition however, they show that urinary lossesof vitamin B_{12} also depend on the level of cortisone in the body. In the vitamin-deficient rats, 2 mg. of cortisone doubled and 4 mg. tripled urinary vitamin B_{12} losses. In the rats which received only a normal vitamin B_{12} intake, there was no increase in urinary vitamin B_{12} until 4 mg. of cortisone were injected daily. These results are believed to reflect an interference by cortisone of carbohydrate utilization, hence reducing the retention of vitamin B_{12} in the body.

Experiment VIII. Effects of Alloxan-diabetes, Cortisone and Vitamin B_{12} on Excretion of Radioactive Vitamin B_{12}.

Purpose

In this experiment it was particularly desired to study the excretion of vitamin B_{12} in alloxan-diabetic rats fed a vitamin B_{12}-deficient or--adequate ration. In addition, further data were obtained on the effects of cortisone on the urinary excretion of vitamin B_{12}.

Methods

Some of the rats employed in Experiment III were used in this study after having been treated as previously described for 32 days. Five rats from each group were injected intraperitoneally with a dose of 0.1 mcc. of radioactive Co^{60}-labeled vitamin B_{12} before they were placed in the metabolism cages. Twenty-four-hour urine specimens were collected and the radioactivity of each urine sample was determined by the same procedure employed previously.

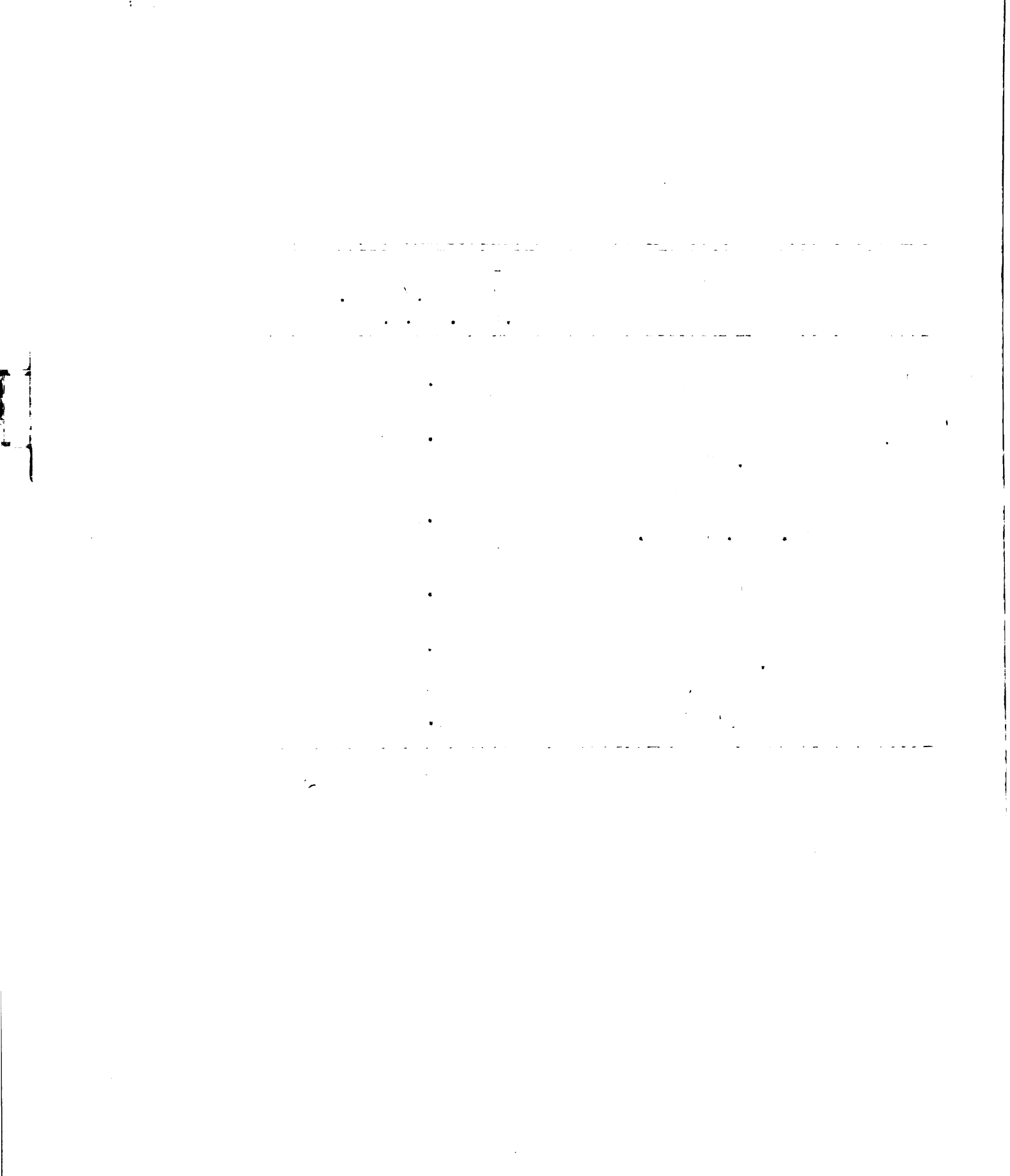
Results

It can be seen in Table X that the vitamin B_{12}-deficient animals (Group 1) retained more of this vitamin than the vitamin B_{12}-adequate rats (Group 2), as was found in the previous experiments. The alloxan-diabetic, vitamin B_{12}-deficient rats (Group 3) retained more while the alloxan-diabetic, vitamin B_{12}-adequate rats (Group 4) retained less

TABLE X

EFFECTS OF ALLOXAN, CORTISONE AND VITAMIN B_{12} ON EXCRETION OF RADIOACTIVE VITAMIN B_{12} IN URINE

Group	Treatment	Co^{60}-Vitamin B_{12} in urine cps/100 gm./24 hr. 0.1 uc. I.P.
1	No vitamin B_{12}	2.6674
2	Vitamin B_{12} 200 mcg./kilogram	4.3936
3	Alloxan 17.5 mg./100 gm.	2.7735
4	Alloxan + vitamin B_{12}	4.6286
5	Cortisone 4 mg./day	6.3852
6	Cortisone + vitamin B_{12}	4.5790



of this vitamin. The amounts of vitamin B_{12} found in the urine were similar to those of the corresponding first two groups. Cortisone increased the excretion of vitamin B_{12} in the urine of the vitamin B_{12}-deficient rats (Group 5), but not in the vitamin B_{12}-adequate animals (Group 6). This is in agreement with the previous experiments.

Conclusions

Alloxan did not alter the urinary excretion of vitamin B_{12} in either the vitamin B_{12}-adequate or -deficient rats. This indicates that alloxan-diabetic rats can utilize vitamin B_{12} as well as normal rats, and is in agreement with the other data in Experiment III showing that in alloxan-treated rats, vitamin B_{12} can increase food intake, efficiency of food utilization and body weight gains. It is concluded therefore, that vitamin B_{12} can act independently of insulin insofar as the foregoing effects are concerned.

Experiment IX. Effects of Glucose Administration on Excretion of Vitamin B_{12} in Alloxan, Cortisone and Vitamin B_{12}-Treated Rats

Purpose

In this experiment, it was desired to ascertain the pattern of vitamin B_{12} excretion in the urine after glucose administration to normal, alloxan-diabetic and cortisone-treated rats. It was hoped that this would provide further information on vitamin B_{12} metabolism as influenced by the foregoing treatments.

Methods

At the end of 35 days of treatment, five rats from each group in Experiment III were starved for 12 hours and were injected intraperitoneally with a dose of 750 mg. of dextrose in 5 ml. of physiological saline. This was immediately followed by another intraperitoneal injection of 0.1 mcc. of radioactive Co^{60}-labeled vitamin B_{12}. The rats were placed in the metabolism cages for 24 hours for urine collection, and the radioactivity in each urine sample was counted.

Results

The results are shown in Table XI. Approximately the same amounts of vitamin B_{12} was excreted in the urine of the vitamin B_{12}-deficient rats (Group 1), as in previous experiments. However, much less vitamin B_{12} was excreted in the

TABLE XI

EFFECTS OF GLUCOSE INJECTIONS ON VITAMIN B_{12} EXCRETION IN ALLOXAN, CORTISONE AND VITAMIN B_{12}-TREATED RATS

Group	Treatment	Co^{60}-vitamin B_{12} in urine cps/100 gm./24 hrs.	
		Results from table (Without glucose) X	Present results
1	No vitamin B_{12}	2.6674	2.3276
2	Vitamin B_{12} 200mcg./kilogram	4.3936	2.9465
3	Alloxan 17.5 mg./100 gm.	2.7235	2.6827
4	Alloxan + vitamin B_{12}	4.6286	4.5466
5	Cortisone 4 mg./day	6.3852	6.7698
6	Cortisone + vitamin B_{12}	4.5790	2.3504

urine of the vitamin B_{12}-adequate animals (Group 2) than in previous experiments. In Groups 3 and 4, about the same amount of vitamin B_{12} appeared in the urine as in Experiment VIII. The administration of glucose did not alter vitamin B_{12} excretion in the cortisone rats fed the vitamin B_{12}-deficient diet (Group 5), but decreased the loss of vitamin B_{12} in the vitamin-fed animals (Group 6).

Conclusions

The decreased excretion of radioactive vitamin B_{12} in Group 2 is probably a reflection of increased glucose utilization in these rats. Glucose administration did not alter urinary vitamin B_{12} losses in either of the alloxan-treated groups. Perhaps this can be attributed to the fact that blood sugar was already high and was being used to the maximum but limited ability of these animals. Hence the additional injection of glucose did not alter vitamin B_{12} metabolism. The glucose injections also did not alter the loss of the vitamin in the cortisone-treated, vitamin B_{12}-deficient rats, probably because glucose was also being used to the limited maximum in these rats. The presence of large doses of cortisone together with a deficiency of vitamin B_{12} inhibited glucose metabolism. In Group 6 however, extra glucose could be utilized because these rats were receiving vitamin B_{12} in their diet. Hence less vitamin B_{12} was excreted into the urine.

Experiment X. Effects of Insulin Injections on Vitamin B_{12} Excretion in Normal, Alloxan and Cortisone-treated Rats

Purpose

Since there were strong indications that vitamin B_{12} was required for full insulin action in the previous experiments, it was desired to determine the effects of insulin on the excretion of radioactive vitamin B_{12} of normal, alloxan-diabetic and cortisone-treated rats.

Methods

Thirty weanling rats were fed the vitamin B_{12}-deficient stock diet for 60 days. At the end of this period, the rats were divided into six uniform groups of five each and were treated as follows for 30 days:

Group 1. No vitamin B_{12}
2. Vitamin B_{12} -- 200 mcg./kilogram of diet
3. Alloxan -- 17.5 mg./100 grams
4. Alloxan + Vitamin B_{12}
5. Cortisone -- 4 mg./day/rat
6. Cortisone + Vitamin B_{12}

At the end of 20 days, all rats were starved for 12 hours and initial blood samples were collected for glucose determinations. Three doses of 0.5 unit of insulin were injected into each rat at eight-hour intervals during a period of 24 hours. A dose of 0.1 mcc. of radioactive Co^{60}-labeled vitamin B_{12} was injected following the first insulin

injection into each rat, and all injections were by the intraperitoneal route. Urine samples were collected for 24 hours and radioactivity was determined as before. Food was withheld during this period.

Results

The results are shown in Table XII. During the period of insulin administration the rats in Groups 1 and 2 were in a semi-conscious condition because of hypoglycemia, and consequently the data from these rats may not be entirely valid. The rats of the other four groups were not adversely affected by the insulin injections. Insulin apparently produced retention of the injected radioactive vitamin B_{12} in all groups, since less of this vitamin appeared in the urine than was found under similar treatment but without insulin injections in Experiment VIII. It will be noted that the decrease in vitamin B_{12} excretion in Group 5 was much less than in the other groups.

Conclusions

When the results of Table XII are compared with Table X it can be seen that insulin decreased the urinary losses of vitamin B_{12} in all groups irrespective of previous treatment. This seems logical since the foregoing experiments have indicated that insulin increases vitamin B_{12} requirements by the body. The particularly small percentage decrease in

TABLE XII

EFFECTS OF INSULIN INJECTIONS ON EXCRETION OF RADIOACTIVE VITAMIN B_{12} IN NORMAL, ALLOXAN AND CORTISONE-TREATED RATS

(0.5 u. of insulin/rat at 8-hour interval)

Group	Treatment	Co^{60}-vitamin B_{12} in urine cps/100 gm./24 hrs.		
		Results from table X (Without insulin)	Present results	Percentage decrease
1	No vitamin B_{12}	2.6674	1.2514	53.09
2	Vitamin B_{12} 200 mcg./kilogram	4.3936	1.3698	68.82
3	Alloxan 17.5 mg./100 gm.	2.7235	1.4872	45.39
4	Alloxan + vitamin B_{12}	4.6286	1.6270	64.84
5	Cortisone 4 mg./day	6.3852	4.8345	24.28
6	Cortisone + vitamin B_{12}	4.5790	1.4689	67.92

vitamin B_{12} excretion seen in the cortisone-treated rats on the vitamin-deficient diet (Group 5) is believed to reflect a greater insulin resistance in these animals. In short, insulin was less effective in the presence of cortisone, less glucose was utilized and more vitamin B_{12} was excreted into the urine. A further comparison of these two tables shows that in every case, the decrease in vitamin B_{12} excretion was more marked in the vitamin-adequate (Groups 2, 4 and 6) than in the vitamin-deficient rats (Groups 1, 3 and 5). This again appears logical, since insulin was more effective in the vitamin-adequate rats, more glucose was metabolized and hence more vitamin B_{12} was retained in the body. It should be recalled that the rats in Groups 2, 4 and 6 were receiving 200 mcg. of vitamin B_{12} per kilogram of diet, or ten times more than their normal requirements. It is remarkable therefore, that the insulin injections should have altered the excretion pattern of vitamin B_{12} in these rats, since this suggests that insulin increased vitamin B_{12} needs by perhaps ten fold or more. It would be interesting to repeat this experiment but feed only normal vitamin B_{12} requirements.

DISCUSSION

In the reports from this laboratory dealing with interactions between cortisone and vitamin B_{12} (Meites *et al.* 1951, 1952a, 1952b, 1953), it was suggested that large doses of cortisone increased requirements for vitamin B_{12}. This view was based on the findings that (a) large doses of cortisone aggravated the condition of rats on a vitamin B_{12}-deficient diet, as indicated by inhibition of body and hair growth, decreased appetite and increased nitrogen losses in the urine; and (b) when normal intake of vitamin B_{12} was permitted, it had relatively little or no ability to overcome the catabolic actions of cortisone, and an intake at least ten times greater than normal was necessary to produce any marked counteraction of cortisone. It was also observed that while vitamin B_{12} increased appetite and the efficiency with which food could be converted into body weight gains, it was prevented from doing so to its fullest capacity by cortisone. It became of primary interest therefore, to attempt to discover why large doses of cortisone increased vitamin B_{12} needs.

The results presented in this study and the related observations of other workers are believed to provide some answers to the above questions. First it has been shown that large doses of cortisone increase the secretion of insulin

by the pancreas of the rat and guinea pig (Franckson et al. 1953, Hausberger et al. 1953). Second, insulin increases the need for vitamin B_{12}, as demonstrated in the present experiments. Thus, (a) insulin was more effective in reducing blood glucose in the presence of adequate vitamin B_{12} than on a deficient diet, (b) insulin reduced the excretion of radioactive vitamin B_{12} in the urine, and (c) injected glucose was more easily metabolized in vitamin B_{12}-adequate than in vitamin B_{12}-deficient rats. Third, cortisone produces insulin resistance, as was observed here and by others (Franckson et al. 1953). This is believed to further increase the need for vitamin B_{12}, since even in the presence of cortisone vitamin B_{12} is able to augment the effectiveness of insulin. Fourth, it was confirmed that large doses of cortisone increase the urinary losses of vitamin B_{12}, particularly in rats whose diet is deficient or just meets normal needs for vitamin B_{12}. Whether this represents a direct effect of cortisone on vitamin B_{12} metabolism, an effect on the kidneys or circulation, or a change in the metabolism of carbohydrate can not be adequately answered at present. Most of the evidence in this thesis favors the latter possibility.

Large amounts (ten times normal) of vitamin B_{12} were able to partially overcome the catabolic actions of excessive doses of cortisone under ad libitum feeding but not on limited food intake. Apparently, these effects of the

vitamin were produced by inhibiting gluconeogenesis from protein by cortisone and by increasing glucose utilization. This depended on the ability of the vitamin to increase food intake. Long et al. (1940) and Engel (1949) similarly observed that administration of large amounts of carbohydrate to rats injected with adrenal cortical hormones counteracted the protein catabolic action of the latter. The observation that vitamin B_{12} was ineffective against cortisone under limited feeding conditions is in agreement with similar findings by Rupp and Paschkis (1953). Indeed it has been noted that vitamin B_{12} does not elicit any growth effect in rats when food intake is limited (Rupp et al. 1951; Baker, 1953). Since vitamin B_{12} is concerned principally with carbohydrate metabolism (Bosshardt et al. 1950; Chow et al. 1952; Black et al. 1952), it appears likely that the decreased ability of cortisone to induce gluconeogenesis from protein in the presence of vitamin B_{12} is by way of a direct action on carbohydrate metabolism. This remains to be elucidated.

Large doses of cortisone partially interfered with the ability of vitamin B_{12} to increase the efficiency of food utilization for body growth. This is believed to be due in part to the loss of sugar in the urine and in part to increased insulin resistance, both of which were demonstrated in the present study. Although insulin does not appear to be essential for vitamin B_{12} function, as seen in the

alloxan-diabetic rats, it may act synergistically with the vitamin in favoring lipogenesis from glucose. The latter has been shown to be an independent function of both substances. Thus cortisone, by decreasing the effectiveness of insulin, would depress its synergistic action with vitamin B_{12}. Studies of fat formation from labeled glucose will help to determine whether this is actually true.

The fact that in alloxan-diabetic rats vitamin B_{12} was albe to increase food intake, efficiency of food utilization for body growth and glucose metabolism deserves further comment. It will be recalled that vitamin B_{12} was practially as effective in these respects in the alloxan-treated as in the normal rats. Sturtvant et al. (1954) have similarly reported that when food intake was increased in alloxan-diabetic rats, there was increased hyperglucosuria accompanied by greater body growth. These observations are of considerable interest since it has been claimed that diabetic animals have practically no capacity to convert glucose into fat (for review of lipogenesis in diabetes see Gurin, 1954). Unfortunately no direct measures of lipogenesis were made in the present work, and a carcass analysis of the alloxan-diabetic rats would have been particularly informative. If it is presumed, however, that vitamin B_{12} did favor lipogenesis in these rats, the possibility arises that the vitamin, particularly in large doses, can substitute

for one of essential functions of insulin. This deserves further study.

There is evidence that vitamin B_{12} may not only be able to function independently of insulin, but also of the adrenal cortical hormones. Meites (1953) and Ralli et al. (1952) found that excessive doses of vitamin B_{12} were able to maintain life and partial body growth in adrenalectomized rats. This was accompanied by increased food intake and efficiency of food utilization. Cortisone and insulin may also be able to function in the absence, or at least in the presence of only a limited intake of vitamin B_{12}. It is clear that the functions of these two hormones are easily modified by the concentration of vitamin B_{12} in the diet, and it is regretable that no intermediate levels of vitamin B_{12} were used in the present study. However, it was shown that on a vitamin B_{12}-deficient diet, insulin was less effective in metabolizing glucose and cortisone was more effective in producing gluconeogenesis from protein. The reverse was true on a vitamin B_{12}-adequate diet. This is believed to demonstrate the importance of vitamin B_{12} in maintaining normal carbohydrate metabolism under the delicate but opposing actions of cortisone and insulin on blood sugar levels.

Vitamin B_{12} is perhaps more important in cortisone-insulin interactions than other B-vitamins, although little information is yet available of the relation of other vitamins

to these two hormones. It has been noted, however, that a "vitamin B-complex deficiency" produces insulin resistance in animals and human beings (Martin, 1937; Elsonn et al. 1940; Biskind, 1945; Samuels, 1948) and that large doses of cortisone may aggravate the condition of young rats on a thiamin-deficient diet (Wilwerth and Meites, 1953). The need for further studies with other B-vitamins is clearly indicated, since there is ample evidence that each does not have the same role in carbohydrate, fat or protein metabolism.

In conclusion, it has been demonstrated that cortisone, the pancreas and vitamin B_{12} all interact on carbohydrate, protein and vitamin B_{12} metabolism. A change in the body level of any one of these factors modifies the function of the other two. It is believed that further studies of these and other vitamin-hormonal inter-relationships will increase our understanding of the intricate machinery of the body, and may even lead to more rational and effective hormone and vitamin therapy in man and animals.

SUMMARY

1. When young rats were fed a vitamin B_{12}-deficient diet, supplementation with this vitamin increased appetite and body weight gains, slightly increased blood glucose, greatly increased glucose tolerance, but slightly decreased urinary nitrogen excretion. When one to four mg. of cortisone acetate daily were injected into vitamin B_{12}-deficient rats, there was a progressive increase in urinary nitrogen, increased hyperglycemia and glucosuria, decreased glucose tolerance, reduced body weight gains and decreased appetite. When 200 mcg. of vitamin B_{12} per kilogram of diet was fed to cortisone-injected rats, and they were permitted to eat ad libitum, increases in urinary nitrogen losses were largely prevented, the hyperglycemia and glucosuria were reduced, glucose tolerance was increased and body growth was increased. Vitamin B_{12} was ineffective in these respects when food intake was restricted to that of animals receiving cortisone without vitamin B_{12}. It is concluded that large doses of vitamin B_{12} can partially counteract the protein catabolic actions of cortisone by increasing appetite, increasing the availability and utilization of carbohydrate by the organism and reducing gluconeogenesis from protein.

2. Large doses of cortisone partially interfered with the favorable action of vitamin B_{12} in increasing the

efficiency of food utilization for body growth. This was accompanied by hyperglycemia and glucosuria, and was related to increased insulin resistance. Less carbohydrate was therefore left available for transformation into body weight gains (probably fat).

3. Alloxan-diabetes reduced body growth and the feed/gain ratio on the vitamin B_{12}-deficient but not on the vitamin B_{12}-adequate diet. In the latter rats there was much higher blood glucose, more urinary glucose, increased glucose tolerance but about the same urinary nitrogen losses as in the former animals. It is concluded that vitamin B_{12} can act independently of insul insofar as its effects on glucose utilization and body growth are concerned.

4. Single injections of insulin (2 units in most cases) were much more effective in reducing blood glucose in normal, alloxan-diabetic and cortisone-treated rats on a vitamin B_{12}-adequate than on a vitamin B_{12}-deficient diet. This indicates that an ample supply of vitamin B_{12} is essential for maximum insulin action. By far the greatest resistance to insulin was found in the cortisone-treated rats on the vitamin B_{12}-deficient diet, confirming the findings that cortisone increases insulin resistance.

5. (a) Injections of large doses of cortisone (2 to 4 mg. daily) increased the urinary excretion of radioactive vitamin B_{12}, particularly in rats fed a vitamin B_{12}-deficient diet. On a diet meeting only normal requirements for

vitamin B_{12} (20 mcg./kilogram), cortisone did not increase urinary vitamin B_{12} until 4 mg. were injected daily. In general, the amounts of radioactive vitamin B_{12} lost in the urine were shown to be directly related to the dose of cortisone administered.

(b) Intraperitoneal injections of 750 mg. of glucose did not change urinary losses of vitamin B_{12} in alloxanized rats fed either a vitamin B_{12}-adequate or -deficient diet. Apparently blood glucose was already being used to the maximum extent possible in these rats.

(c) In normal and cortisone-treated rats on a vitamin B_{12}-adequate but not on a vitamin B_{12}-deficient diet, intraperitoneal injections of glucose decreased the loss of urinary vitamin B_{12}. This is believed to reflect greater glucose utilization in the former animals.

(d) Insulin injections (3 injections of 0.5 unit each in 24 hours) greatly reduced urinary radioactive vitamin B_{12} losses in normal, alloxanized and cortisone-treated rats whether on a vitamin B_{12}-adequate or -deficient diet. This is believed to reflect greater glucose utilization in these animals. The decreases in urinary vitamin B^{12} were less on the vitamin-deficient diet, particularly in the cortisone-treated animals, and is believed to reflect the reduced effectiveness of insulin on glucose utilization in these rats.

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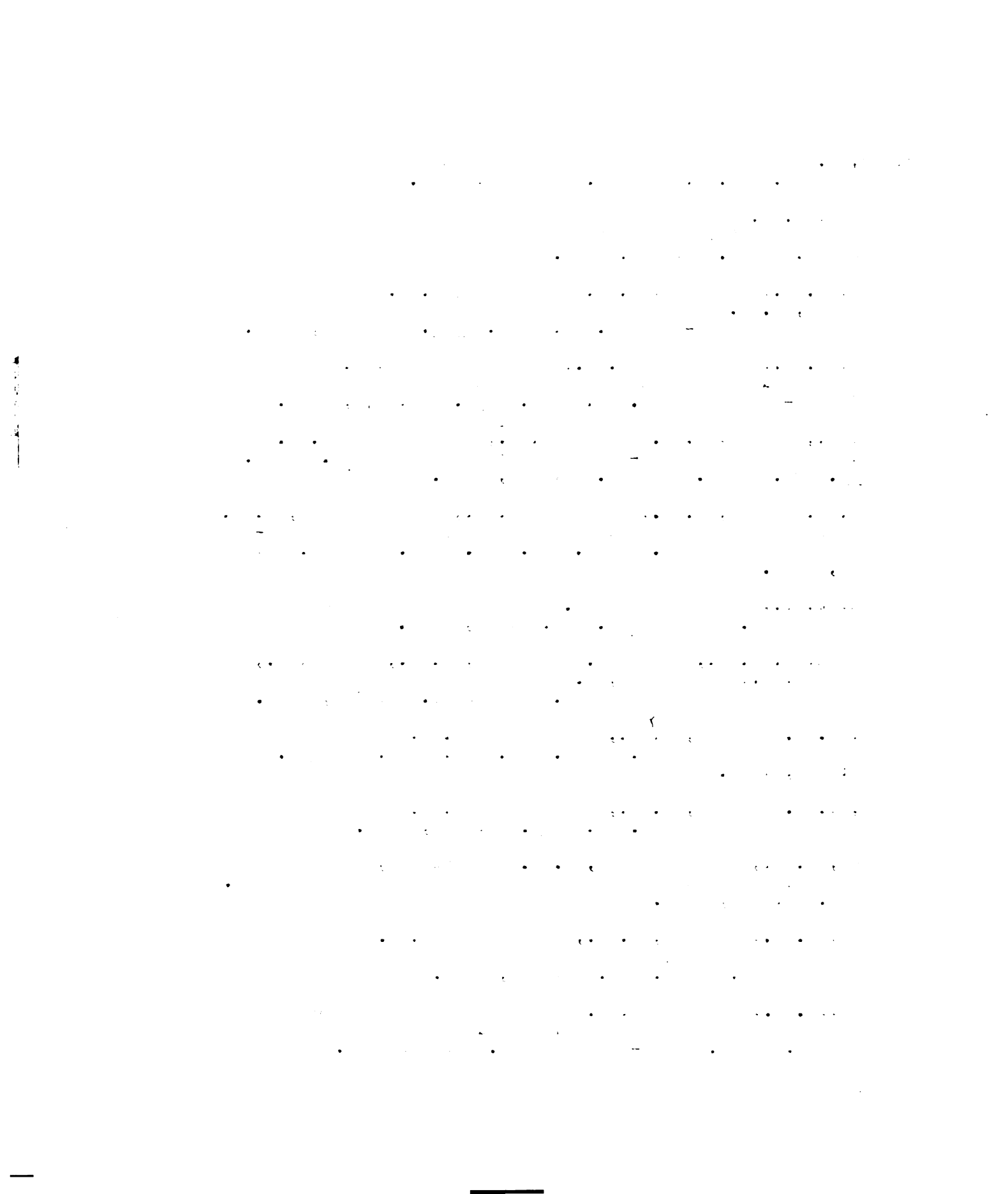

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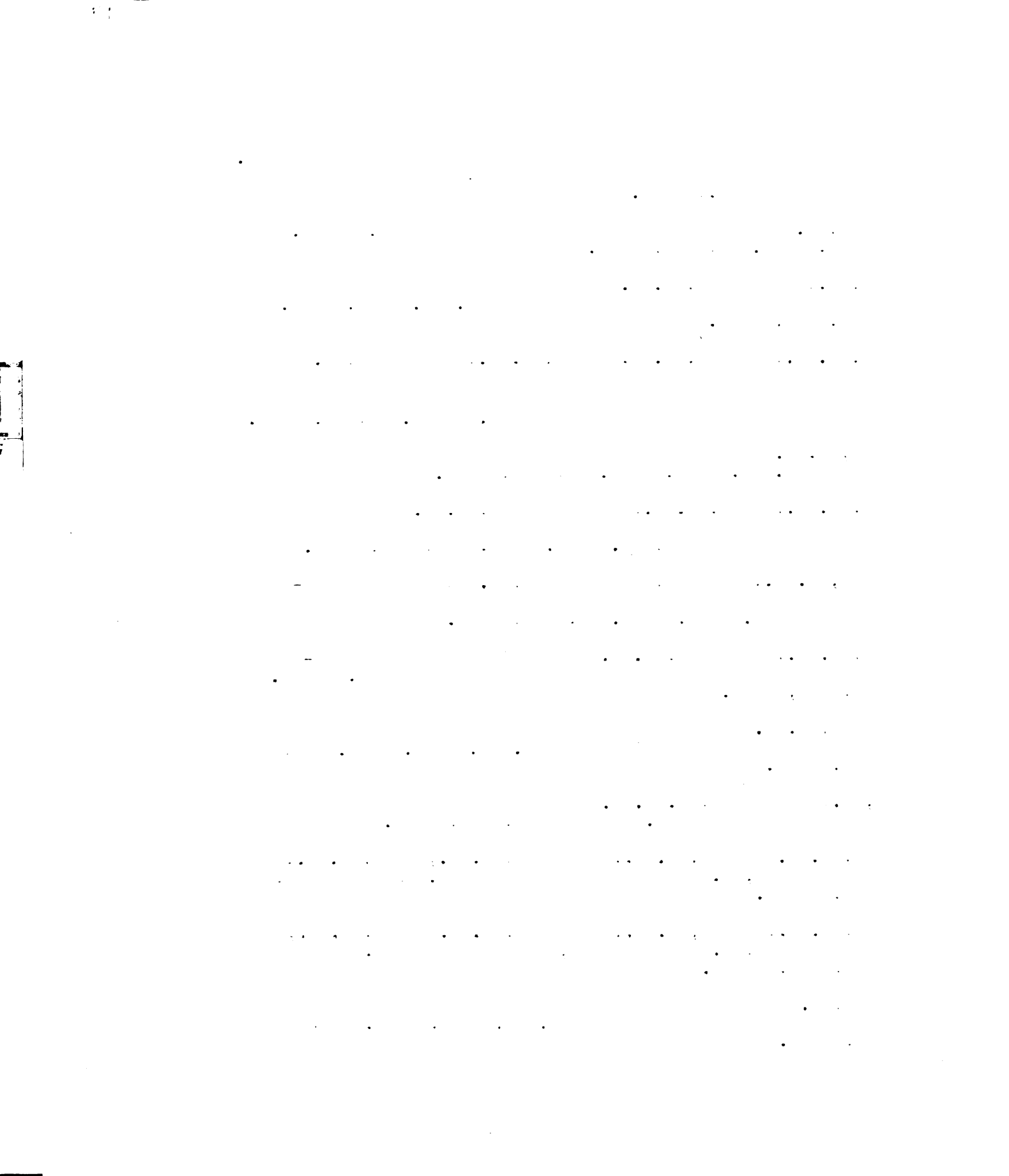

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APPENDIX

1. Blood Glucose Levels after a Single Injection of Insulin in Alloxan-diabetic Rats

Purpose

Preliminary to the experiments in which insulin was used, it was important to determine at what period of time blood glucose would fall to the lowest level after a single injection of insulin.

Methods

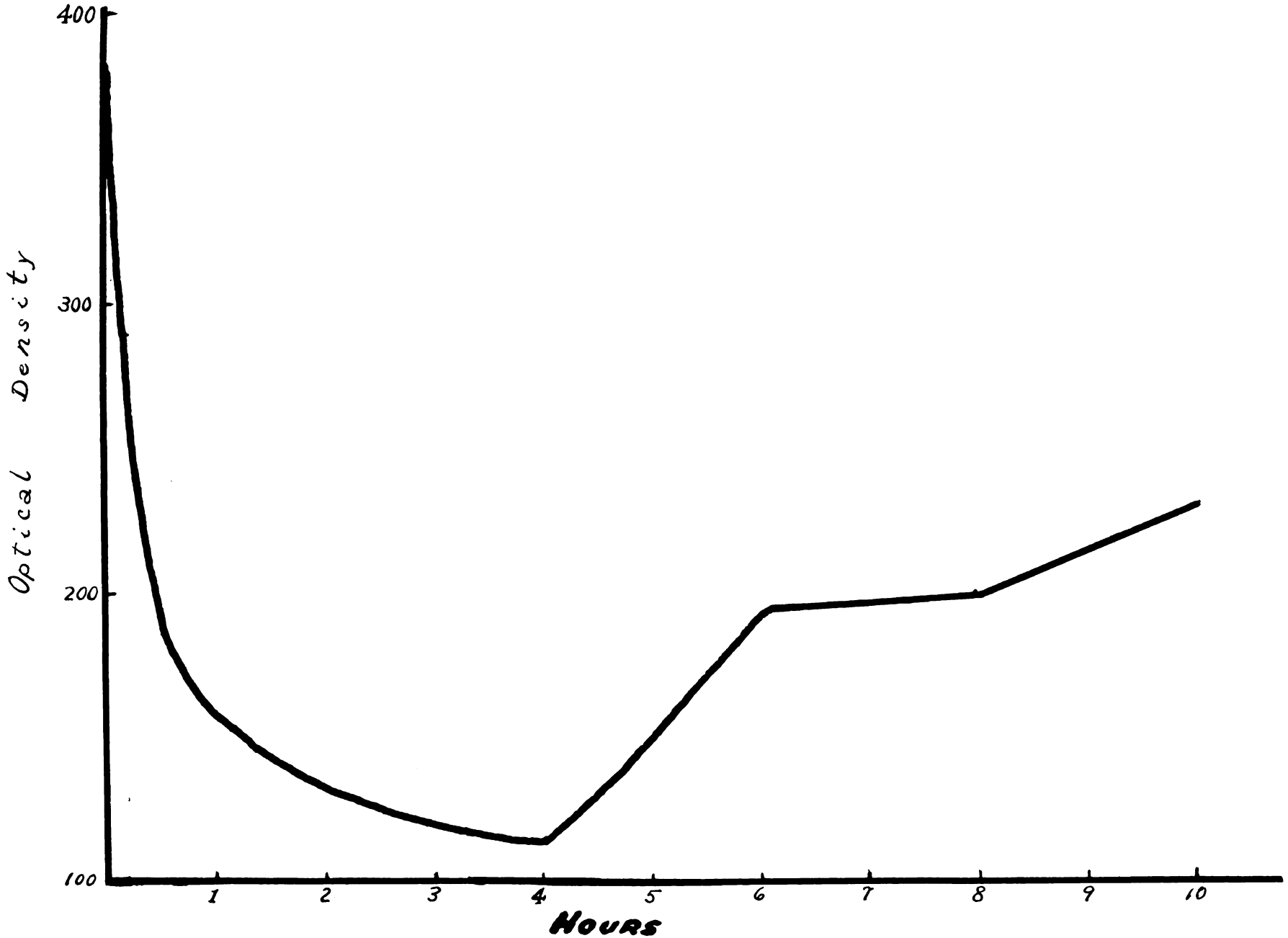
Eight adult male rats were used in this experiment. They were starved for 72 hours, only water being permitted during this time. At the end of this period, a dose of 17.5 mg. of alloxan monohydrate per 100 grams of body weight was injected subcutaneously (Bailey, 1949). Diabetes was definitely established five days after the injection as indicated by a marked hyperglycemia. It was found that blood glucose values were between 320 mg. to 400 mg. percent. Blood glucose was determined by the Hartman, Shaeffer and Somogyi micromethod (Hawk et al. 1951). A volume of 0.2 ml. of blood was collected with a Folin-Wu micropipette from the tail of the rat, after first cleaning it with 80 percent alcohol, and then cutting off the tip. Two units of insulin were injected intraperitoneally into each rat. Food was removed 12 hours prior to the injection. After this, blood was collected every two hours for glucose determinations. Food was withheld during the collection period.

Results

Figure 8 shows that the average initial blood glucose level was 380 mg. percent. After insulin administration, blood glucose fell gradually reaching the lowest level of about 125 mg. percent in four hours. Blood glucose returned to 250 mg. percent 10 hours after the insulin injection.

Conclusions

These data showed that with the dose employed, the greatest insulin effect could be expected four hours after injection. Consequently this time interval was used in all experiments in which insulin was employed.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2
3
4
5
6
7
8
9
10
HOURS

2. Blood Glucose Determinations

a. Somogyi-Shaffer-Hartman Method (Hawk et al. 1951)

A volume of 0.2 ml. of blood was drawn from the tail of each rat by a Folin micropipette and was mixed into 5.8 ml. of water in a 25-ml. Erlenmeyer flask; the pipette was then rinsed several times with the lacking water. A volume of one ml. of 1.8 percent of zinc sulfate and one ml. of 0.1 N of sodium hydroxide were added and mixed. The flask was stoppered, shaken and the contents were filtered through No. 1 dry filter paper.

Five ml. of the Shaffer-Hartman copper reagent were measured in a 25 x 250 mm. test tube, and 5 ml. of the blood filtrate was mixed into it, shaken, and covered with a glass bulb. The test tube was placed in a boiling water bath for 15 minutes. It was then cooled, 1 ml. of 5 N sulfuric acid was added, and it was titrated with 0.005 N sodium thiosulfate. Starch was used as an indicator. A blank was run on 5 ml. of the copper reagent after boiling with an equal amount of water. In the calculations, the blank titration was subtracted from the titration of the unknown. This gave the ml. of thiosulfate required for the unknown. For the glucose equivalent, the Table (page 525) in Hawk et al. (1951) was consulted. Since this table applies to the usual 1:10 dilution of blood, and in the present case, a 1:40 dilution was used, the mg. of glucose in 100 ml. of blood given in the table were multiplied by four.

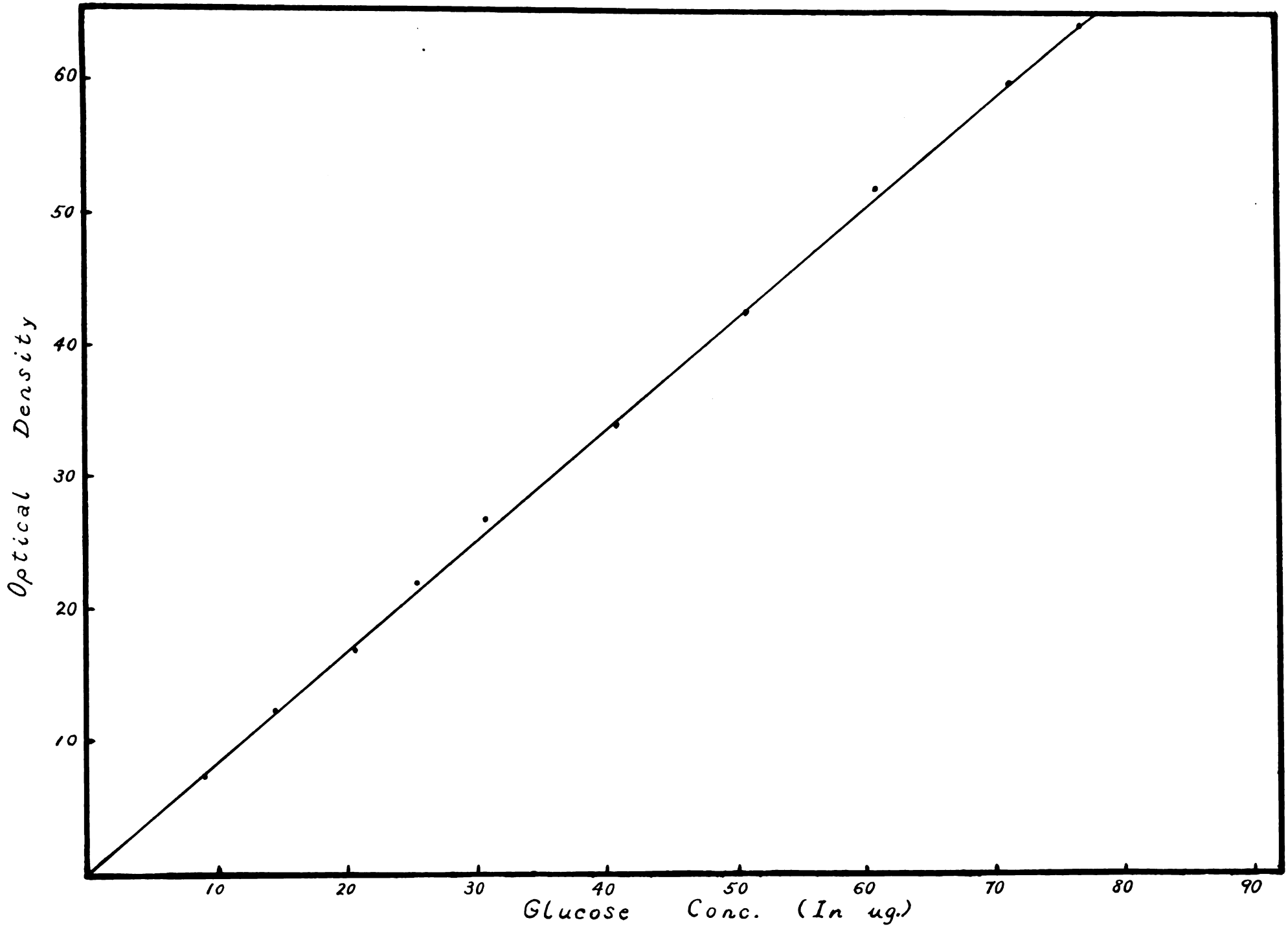
b. Folin and Malmros Method (Hawk et al. 1951)

With a Folin micropipette 0.1 ml. of blood was drawn from the tail of a rat and was transferred to a centrifuge tube containing 10 ml. of dilute tungstic acid. This was mixed and centrifuged. Four ml. of the water-clear supernatant fluid were transferred to a test tube graduated at 25 ml. To this, 2 ml. of 0.4 percent potassium ferricyanide-carbonate solution were added. The contents were heated in boiling water for 15 minutes and cooled in running tap water for 2 minutes. Then 5 ml. of ferric iron solution were added and mixed. Two minutes afterwards, the contents were diluted with water almost to the 25-ml. mark, two drops of alcohol were added to prevent foaming, and water was added exactly to the 25-ml. mark and mixed. It was read in a Fisher electrophotometer 20 minutes later. A green plate filter of 525 mu. wavelength was used. The photometer was initially set to zero density with water.

For preparation of the standard solution of sugar, a stock solution of 1 percent glucose was made up in saturated benzoic acid. The stock solution was diluted to 0.01 mg. to 0.1 mg. per 0.1 ml. of water. The optical density for each amount was read on the photometer, and a linear line was drawn from these values.

The calculation of blood glucose was as follows:

$$\text{mg. percent glucose} = \frac{\text{density of unknown}}{\text{density of standard}} \times 0.04 \times \frac{10}{4.0} \times \frac{100}{0.1}$$


Optical Density
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
Glucose Conc. (In ug.)

3. Determination of Total Urinary Nitrogen

Koch and McMeekin Method (Hawk et al, 1951)

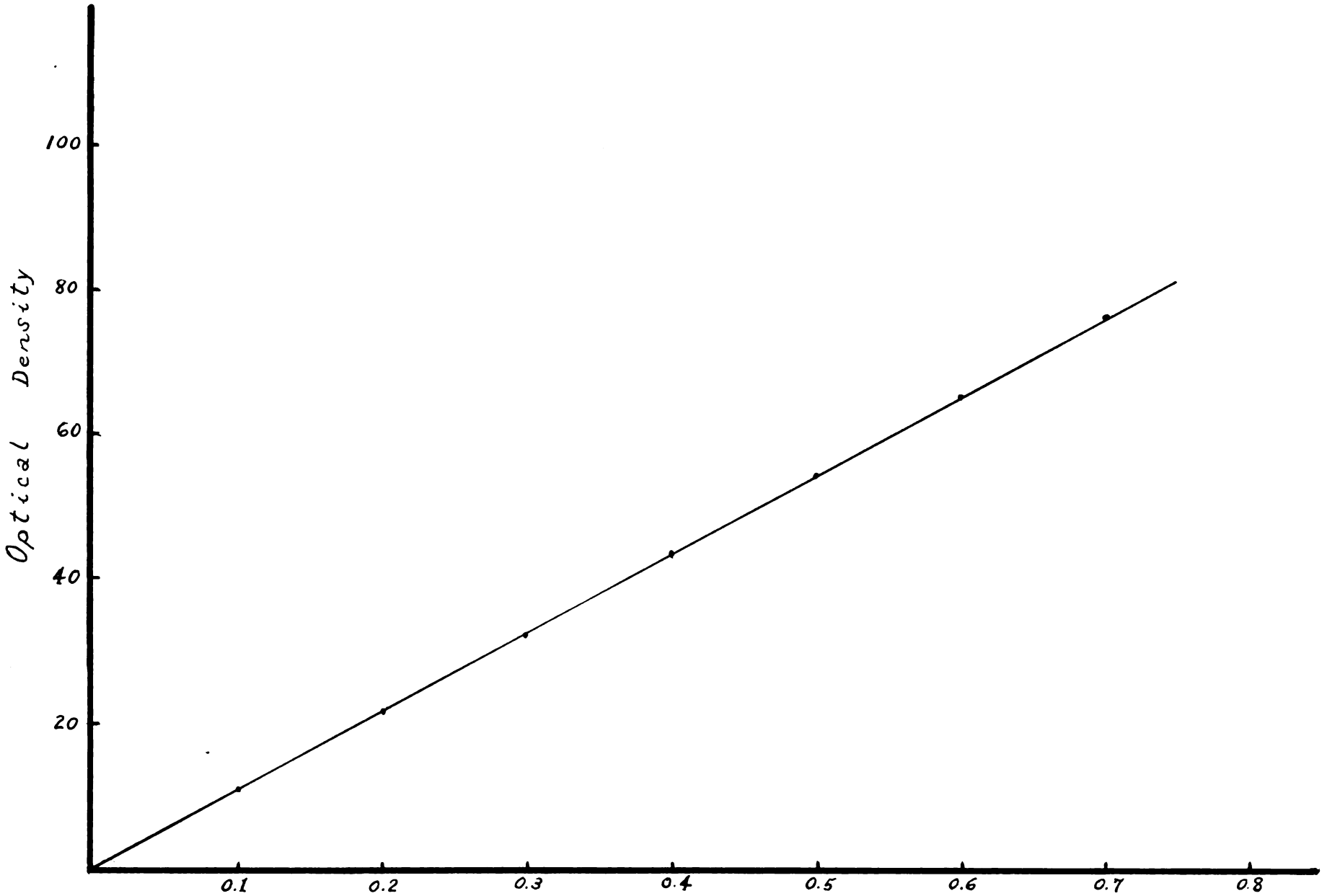
One ml. from a 24hour urine specimen was diluted to 50 ml. and mixed in a volumetric flask. One ml. of the dilute solution was pipetted into a micro-Kjeldahl flask, and one ml. of 50 percent sulfuric acid was added and mixed. The flask was heated over a gas flame under a hood for 10 minutes, after which 3 drops of 30 percent hydrogen peroxied were added. The flask was heated 6 more minutes until all the sulfuric acid fumes disappeared. It was then cooled for 30 minutes and diluted to 75 ml. with water. A total of 15 ml. of Nessler's reagent was added and the whole was diluted to 100 ml. This was left to stand for 10 minutes and was then read on a Fisher electrophotometer in which a green plate filter of 525 mu. wavelength was used.

For a standard nitrogen preparation, 0.0714 gram of ammonium sulfate was dissolved in one liter of water together with a few drops of concentrated sulfuric acid as a preservative. This contained 1 mg. of nitrogen per 10 ml. It was used in amounts of 0.1 ml. per 1 ml. of the stock solution, and was diluted with 15 ml. of Nessler's reagent and water to 100 ml. The values were read on the photometer and a linear line was drawn.

The calculations were as follows:

$$\frac{\frac{\text{reading of standard}}{\text{reading of unknown}} \times \text{mg. N in standard} \times \text{urine volume}}{\text{body weight}}$$

Total nitrogen was expressed as mg./100 gm. body weight/24-hour urine specimen.


Optical Density
100
80
60
40
20
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

4. Determination of Urinary Glucose

Hawkins and Van Slyke Method (Hawk et al. 1951)

One ml. of the 24-hour urine specimen was diluted to 50 ml. in a volumetric flask, and 2 ml. of this was pipetted into a pyrex test tube (14 x 125 mm.). Two ml. of ferricyanide solution were added and mixed. The flask was immersed in a beaker of boiling water containing a similar test tube with water alone for comparison. A white background was made on the sides of beakers into which the solutions were poured by pasting on white paper. The time in seconds required for the last trace of yellow to disappear was determined with a stopwatch. From the chart on page 865 of Hawk et al. (1951), estimations of glucose in gm./100 gm. body weight/24-hour urine were calculated, i.e.:

$$\frac{\text{gm. of glucose x 50 x urine volume}}{\text{body weight}}$$

4 Kilogram Stock Soybean Meal Diet

Yellow corn meal (Thoman)	1400 gm.
Ground whole wheat (Thoman)	1000 gm.
Alfalfa leaf meal (Thoman)	240 gm.
Brewer's yeast (Strain G) (A. Busch)	120 gm.
Iodized salt	40 gm.
Soybean meal (low fiber, solvent extracted, containing 50% protein, Archer-Daniels-Midland)	1200 gm.