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An Effect of D-Biotin on Rat Liver Glucose Utilization

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AN EFFECT OF d-BIOTIN ON RAT LIVER GLUCOSE UTILIZATION

by

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LIFE

John C. Coggeshall was born in Chicago, Illinois, on July 16, 1939. In June, 1957, he graduated from Morgan Park High School, Chicago. He received the degree of Bachelor of Science in Biology, in January, 1963, from Illinois Institute of Technology.

In February, 1963, the author began his advanced studies in the Graduate School of Loyola University, Department of Biochemistry and Biophysics. During the intervening period the author has held the position of Research Assistant, with financial support from an NIH Training Grant in Biochemistry.

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CHAPTER I

INTRODUCTION

A. Purpose

The purpose in view in preparing this thesis is to present the experimental data observed in the laboratory which indicates that the vitamin, d-biotin, plays a physiological role in the metabolism of glucose by mammalian organisms. The implication that biotin plays a role in mammalian glucose utilization was initially predicted on the basis of the known biochemical pathways involving glucose coupled with an analysis of the biochemical lesions which typify diabetes mellitus. Since the working hypothesis which evolved from this analysis is to some extent subjective in nature, and therefore in itself open to valid criticism, it is also presented as an integral part of the thesis.

B. General Theory

The physiological role of D-glucose would appear to be two-fold:

1. As a source of energy, either directly as glucose or indirectly through hydrolysis of glycogen, its polymerization product.

2. As a source of carbon atoms for the synthesis of amino acids and fatty acids, and therefore of de novo protein, and triglyceride synthesis.

In regard to the mechanism by which these roles are accomplished, glucose is enzymatically broken down to pyruvate through the well-known glycolytic pathway. Pyruvate has two main pathways open to it for further metabolism:

1. Irreversible decarboxylation to acetyl-coenzyme A, and therefore to a source of carbon atoms for fatty acid synthesis or for further oxidation to carbon dioxide and water with the concomitant production of adenosine triphosphate.

2. Reversible carboxylation to a four carbonketo dicarboxylic acid and therefore as a source of carbon atoms for condensation with acetyl CoA, or as a source of carbon atoms for the de novo synthesis of amino acids having four or more carbon atoms.

Acetyl-CoA, the decarboxylation product of pyruvate, also has two main pathways open to it for further metabolism:

1. Condensation with oxaloacetate as mentioned above.

2. Reversible carboxylation to malonyl-CoA, a three carbon dicarboxylic acid which has been shown to be an absolute requirement for de novo fatty acid synthesis.

Acetyl-CoA, also has two other quantitatively minor, but physiologically significant pathways of metabolism available to it:

1. De novo synthesis of cholesterol.

2. Synthesis of the socalled "ketone" bodies: acetoacetic acid, β-hydroxy butyric acid, and acetone.

It should be noted that carbon dioxide fixation plays a direct role in the de novo synthesis of both protein and triglyceride and also would appear to play an important role in maintaining a continuous supply of oxaloacetate for citric acid cycle operation particularly when an organism is in a state of positive nitrogen equilibrium and amino acids derived from protein hydrolysis are not a major source of dicarboxylic acids.

In light of the above, admittedly simplified, concept of general metabolism let us postulate the existence of an obese sedentary mammalian organism which for some reason has lost the ability to utilize carbon dioxide; the organims, being obese, has significant quantities of adipose tissue which contain stored triglyceride. The early work of Schoenheimer (1) has established that the triglyceride stores of adipose tissue are in a state of dynamic equilibrium with their precursor, acetyl-CoA. We therefore have a situation where triglycerides are being broken down to two-carbon acetyl-CoA units without a concomitant resynthesis of fatty acids

due to the organism's inability to synthesize malonyl CoA through carbon dioxide fixation of acetyl-CoA. All other things remaining equal, such a process must result in a new increase in acetyl-CoA units and a corresponding decrease in total body triglyceride level. The extra acetyl-CoA formed by this process may be handled in any of three remaining ways as discussed earlier. However, since carboxylation of pyruvate to yield oxaloacetate can no longer occur, the level of oxaloacetate must decrease unless there is a corresponding increase in deamination of amino acids yielding Kreb cycle dicarboxylic acids. At any rate, the increasing levels of acetyl-CoA units due to failure of fatty acid synthesis, coupled with the decreasing supply of oxaloacetate from pyruvate, tend to make doubtful the postulate that our hypothetical sedentary organism could divert the increasing levels of acetyl-CoA units wholly by increased citric acid cycle function. The two other main pathways open to acetyl-CoA are cholesterol synthesis and ketone body formation, which might be expected to increase in an effort to normalize the increasing levels of acetyl-CoA formed. The possible net results, then, of our hypothetical organism losing solely the ability to utilize carbon dioxide are:

1. Decreased de novo synthesis of fatty acids.
2. Decreased de novo synthesis of many so called

"nonessential" amino acids and a possible net negative nitrogen balance, in general.

3. Decreased utilization of pyruvate with corresponding decrease in the formation of oxaloacetate and a relative decrease in citric acid cycle function.

4. Increased cholesterol synthesis.

5. Increased "ketone body" synthesis with a corresponding increase in hydrogen ion production.

The above metabolic lesions, if coupled with hyperglycemia, would be remarkably similar to many of the lesions reported to be characteristic of diabetes mellitus (1). Since the vitamin, d-biotin, has been reported by a variety of workers to be directly involved in those carboxylation reactions previously discussed, a review of the available literature pertaining to d-biotin was undertaken. The results of this literature search are reviewed in the following section.

CHAPTER II

LITERATURE REVIEW

Biotin has long been known to be an essential vitamin, but its function has only recently been shown to be that of a bound cofactor in enzymatic reactions involving incorporation or transfer of carbon dioxide (1).

Most of the early literature deals with the *in vivo* or *in vitro* effects of a biotin deficiency caused by feeding large amounts of raw egg white. Egg white contains a basic protein, avidin, which has a very high affinity for biotin and acts as a specific inhibitor for biotin dependent reactions (2). However, it would seem that much of the data garnered from the early reports must be interpreted cautiously since coprophagy was not prevented. That is, due to the fact that the intestinal flora synthesize biotin in rather large amounts (3), and feces represent an additional source of this vitamin to the organism, particularly in the rat in which coprophagy is quite prevalent (4). Nevertheless, the early work is included for completeness, with the added complication that whether

an actual biotin deficiency was present or not, must be kept in mind.

A. Metabolic Function of Biotin

1. Carbohydrate Metabolism of Bacteria and Yeast

The early work in this area deals with the fermentation capacity of various strains of yeast and bacteria which had been demonstrated to require biotin as an essential growth factor.

Williams et al. (4), have shown that a biotin-deprived culture of Saccharomyces cerevisiae will grow moderately well, if the medium contains sucrose. Sucrose is hydrolyzed to glucose-1-phosphate and fructose by sucrose phosphorylase. This led Williams et al., to propose that biotin may control the initial stage of glucose utilization, its phosphorylation by hexokinase (fig. 1). These authors added credence to their postulate, by showing that 2-deoxy-D-glucose which is phosphorylated by hexokinase to 2-deoxy-D-glucose-6-phosphate but is not utilized further, exhibits a greatly reduced rate of phosphorylation in a cell free extract of biotin deficient S. cerevisiae (4).

Furthermore, Strauss and Moat (5) have shown, that the phosphorylation product of fructose, fructose-6-phosphate, or even hexose-diphosphate is independent of a biotin deficiency. These same authors (5),

have also shown that the addition of free biotin in vitro to biotin deficient yeast extract cells restores the weakened hexokinase activity.

However, this apparent relationship between biotin and some initial stage of glucose utilization has not always been demonstrable. Lichstein (6) has shown that in the presence of glucose, a mutant strain of Escherichia coli (23358), when rendered biotin deficient, exhibited growth rates of four times that of the control. It should be stressed at this point, that none of the above experiments have indicated carbon fixation as being a factor in the glucose utilization lesion in the biotin deficient state. Also, there can be little doubt in the above reports as to the existence of an actual biotin deficiency.

2. Carbohyrate Metabolism of the Rat

a. Fasting glucose levels in biotin deficiency.

In more recent work, Wagle (7) has demonstrated that biotin deficient rats have normal fasting glucose levels. Similarly, Terroine (8) has reported that rats deprived of biotin do not show any hyperglycemia, but on the contrary, exhibit a slight hypoglycemia. However, it is not clear from these experiments whether coprophagy was prevented or not. In experiments where coprophagy was prevented, Mistry and Dakshinamurti (9) have reported significant hyperglycemia in all cases under a glucose

load. The hyperglycemia was corrected with insulin administration.

In a similar experiment, but where coprophagy is questionable, the glycemia of biotin-deficient rats, pair fed, and with ad libitum controls, was studied after intraperitoneal injection of 100 mg glucose per 100 gm body weight. The latter group showed the greatest hyperglycemia response, the first group, the least.

As an interesting side point, Terroine (8) has observed that blood pyruvic acid levels in her biotin deficient rats were doubled. This in vivo observation corresponds well with in vitro studies (10, 11) in which various tissue slices or homogenates from biotin deficient mammals exhibited poor utilization of pyruvate.

b. Tissue glycogen and glucose levels.

Terroine (8) has stated that biotin deficiency in no way modifies the glycogen content of liver or muscle. However, Dakshinamurti, et al. (12), have observed a 50% decrease in liver glycogen levels in deficient rats in which coprophagy was prevented. Furthermore, using D-glucose-U-C^{14} incorporation into liver glycogen as an index, they showed that both the specific activity and total radioactivity of liver glycogen was quite markedly reduced. To further illustrate the discrepancy between some of the published data, Gram and Okey (13) found an

increased incorporation of acetate-2-C^{14} into liver glycogen of biotin deficient rats.

Terroine (8) has reported the free reducing sugar level of the liver to be considerably reduced in biotin deficiency. Muscle values remained constant (8).

3. Lipid Metabolism

Rather than present the large volume of evidence reported in the past implicating biotin as playing a role in lipid metabolism, an actual specific role of this vitamin in fatty acid synthesis is presented first. This well-defined role explains (but does not prove) many of the questions raised by the earlier reports.

It was shown, in studies with avian liver, that the conversion of acetyl-coenzyme A (acetyl-CoA) to long chain fatty acids required the presence of adenosine triphosphate, Mn^{+2}, HCO_3^-, reduced triphosphopyridine nucleotide and two protein fractions (14-18). Malonyl coenzyme A was isolated from the reaction mixture by Wakil (19), thus breaking the sequence of reactions leading to fatty acid synthesis into two steps: the formation of malonyl-CoA and its subsequent conversion to palmitic acid. The first of these reactions is catalyzed by acetyl-CoA carboxylase, a conjugated enzyme which contains biotin as a prosthetic

group. Evidence for the participation of enzyme-bound biotin in the synthesis of fatty acids was presented by Wakil, Fitchener, and Gibson (15, 18), who showed that palmitate synthesis from acetyl-CoA was inhibited by avidin. The avidin inhibition was strongly implicated as being due to its biotin-binding site, since incubation of avidin with biotin before its interaction with the carboxylase, relieved this inhibition. The free biotin saturated all of the biotin-binding sites of avidin, and thus eliminated the inhibiting capacity of this protein. This avidin technique is now extensively applied as a diagnostic test for the recognition and participation of biotin-bound enzymes (20-23).

With the above role of biotin in mind, some of the earlier work is presented.

Many workers (24-27) have noted the vicarious role of oleic acid and other "essential" fatty acids in biotin deficient organisms. While there are some discrepancies, it would seem that the participation of biotin in fatty acid synthesis adequately explains the dual role of certain fatty acids in biotin deficiency. The discrepancies encountered in this explanation are those entirely due to measurement of fatty acid levels in certain organs in biotin deficient rats. Guggenheim and Olson (24) have reported more or less identical levels of total fatty acids in

the liver, heart, and blood of biotin-deficient rats in comparison with pair-fed controls (25). Also, on the basis of incorporation of labeled substrate, it has been reported (26, 27) that biotin deficient rats exhibit a normal capacity to synthesize fatty acids. However, Gram and Okey (23) made the following observations after comparing the incorporation rate of acetate-2-C^{14} by biotin-deficient rats and pair-fed controls:

1. The deficient animals excrete the greater part of the acetate-2-C^{14} in the form of $C^{14}O_2$.

2. Acetate-2-C^{14} is only weakly incorporated into liver lipids.

3. As mentioned earlier, preference was shown for the use of labeled acetate in the synthesis of glycogen.

From these results, the authors postulated that there was an inhibition in the synthesis of glycerides in biotin deficiency. As a working hypothesis, these workers suggested, before the work of Waite and Wakil (14-18), that in absence of biotin this inhibition may depend on a decrease in the synthesis of fatty acids.

4. Cholesterol Metabolism

The elucidation of the synthesis of cholesterol was announced only recently (28). It seems quite certain that biotin plays no direct role

in the de novo synthesis of this molecule. However, one interesting effect of biotin deficiency on cholesterol metabolism has been reported which does have some bearing on the postulate presented in this thesis. Biotin deficiency is associated with hypercholesterolemia, as it is observed in the rabbit (29), and the rat (30). While this aberration could be explained in a variety of logical ways, it is also interesting to note that Oxman, and Bell (31) have reported a urinary excretion of ketone bodies in biotin deficiency which is three times the normal.

5. Protein Metabolism

At the present time, there appears to be a great deal of confusion regarding the exact role of biotin in protein metabolism. The issue appears to revolve around three major points:

1. Amino acid synthesis and catabolism.
2. Deoxyribonucleic and ribonucleic acid synthesis.
3. Microsomal synthesis of protein molecules.

Further confusing the issue, is the fact that an aberration in any of the above three areas might possibly lead to a decreased synthesis of any number of enzymes therefore possibly leading to a lack of a key enzyme involved.

a. Metabolism of certain amino acids.

In the catabolism of leucine, one of the intermediates formed is hydroxyisovaleryl-CoA (32). This molecule is then enzymatically carboxylated forming β-hydroxymethylglutaryl CoA (32). This enzyme has been reported by Woesmer (33) to be completely lacking in liver extracts of biotin-deficient rats. No *in vivo* data on the urinary excretion of β-hydroxyisovaleryl-CoA or of leucine has been located. It should be noted that this enzyme is susceptible to avidin inhibition and contains appreciable quantites of bound biotin (34).

To illustrate a drastic aberration in metabolism of an amino acid which does not appear to require biotin as an enzyme bound cofactor, the essential amino acid tryptophan is discussed. The first stage in the degradation of tryptophan involves the formation of formyl-kynurenine. This step does not involve carbon dioxide fixation but nonetheless is completely blocked in *Neurospora crassa*, rendered biotin deficient (35). Also, the biotin deficient rat is incapable of converting an appreciable quantity of tryptophan to niacin derivatives (36). The enzyme involved in the above reaction does not contain biotin and is not inhibited by avidin (37). This, therefore, would seem to indicate, that the protein moiety of the enzyme involved is deficient, abnormal, or absent.

b. Nucleic acid synthesis and function.

While there have been reports of accumulation of various metabolites involved in the synthesis of the purine and pyrimidine bases (38-41), no positive evidence has been presented that indicates biotin involvement in the synthesis of nucleic acids. Moreover, since avidin has not been shown to inhibit the activities of any of the enzymes involved in this process (42), it would appear more logical to conclude tentatively that the synthesis of some of the enzymes involved in DNA and RNA production is at some point inhibited.

In the only recent work done in this area, Mistry (43) has demonstrated abnormalities in both a soluble RNA fraction and a microsomal RNA sediment with a concomitant decrease in the incorporation of C^{14}-labeled amino acid into liver protein in in vitro studied comparing biotin deficient rats with pair-fed controls. This author has also noted that injection of a dicarboxylic acid into the deficient rat previous to sacrifice rendered amino acid incorporation and RNA sedimentation patterns more comparable to normal. From these data, plus the observation that pyruvate is readily carboxylated to oxaloacetate by an avidin inhibited, biotin containing enzyme (44), Mistry has tentatively implied that the primary block is mainly one of synthesis of adequate supplies of

dicarboxylic acid precursors of the so called "non-essential" amino acids (43).

c. Miscellaneous.

In two reports published in 1958-59 (44), a metabolic disturbance in biotin metabolism in human diabetics and alloxan diabetic rats was implied. In a general study of liver disease, the authors noted that in diabetics particularly, the urinary excretion of biotin was markedly increased. Moreover, the injection of biotin intramuscularly was followed by an extra excretion of biotin in an increased amount compared to controls. Moreover, the increase in excretion of biotin following injection was well correlated with the fasting blood sugar. While this may be entirely explained by an osmotic diuretic effect due to an increased blood sugar level, the studies with alloxan diabetic rats point to another alternative. In a time study of the biotin content of liver in alloxan diabetes, the authors noted a sharp decrease in the bound biotin content of their preparations with the final concentration being approximately one-seventh of the control values. These authors report a normal concentration of biotin in liver to be about 3.0 μg/gm. Moreover, injection of insulin prior to sacrifice restored, in large part, to normal, the depressed concentration of bound biotin. While no further work has been reported in

this area, these experiments, plus the variety of reported biochemical lesions in biotin deficiency, give adequate protocol for further investigation.

CHAPTER III

MATERIALS AND METHODS

A. Experiments Involving Crystalline Yeast Hexokinase

Crystalline yeast hexokinase, Grade A, was obtained from California Biochemicals, Inc., California, in 10 mg vials with an estimated total amount of activity of 3300 units. The enzyme was dissolved in 100 ml of ice-cold 0.05 M phosphate buffer, pH 7.4 and frozen until use. The enzyme after six months in the frozen state showed little or no loss of activity. The hexokinase preparation was not dialyzed before use except where indicated in the data section. The assay method of Colowick (46) was used to determine the rate of formation of glucose-6-phosphate. The method involves measurement of the rate of change of optical density, at 560 mμ, due to the change in the ratio of salt to acid of the indicator, cresol red. The theory of the method is described more fully in the data section. The instrument employed was the Beckman Model D spectrophomoter, at a wavelength 520, sensitively 1, 0, slit width 0.1 mm, with the tungsten lamp as a light source. All chemicals

employed were of the highest commercial grade available and were obtained from Sigma Chemicals, Incorporated; and the indicated purity was assumed to be correct. The avidin used was soluble only in 1% sodium chloride. An equivalent amount of sodium chloride was therefore added where needed to all other cuvettes or flasks to insure proper control readings.

B. Experiments Involving Whole Animals

Male albino rats of the Sprague Dawley variety were obtained commercially. The rats were fed a commercial stock diet ad libitum except where indicated. The nutritional status of the rats used is indicated in the data section. Water was given freely. The weight of the rats used was in the 200-250 gram range and is indicated in each experiment. Injections were intraperitoneal, using a 22 gauge needle delivering 0.5 ml of the indicated solution over a period of approximately 30 seconds. Blood at the indicated intervals was collected from a severed tail vein, deproteinized according to the method of Nelson and Somogyi (47) and the glucose present was measured using glucose oxidase. The technique of measurement is described in Section C. The instrument used to measure the optical densities was the Spinco Spectrocolorimeter at a wavelength setting of 410 mμ.

C. Experiments Involving Rat Liver Homogenates

The rats were killed by cervical fracture and decapitated. The livers were removed as quickly as possible and soaked in ice cold 0.05 M potassium phosphate buffer, pH 7.4. The rinsed liver was diced, blotted, and weighed. In all experiments, 1.5 x volume of ice cold 0.05 potassium phosphate buffer, pH 7.4, was then added and the resulting mixture blended gently with a loosely fitting pestle. Ten ml of the homogenous mixture was then centrifuged at 15,000 x G for 30 minutes at a temperature of 0-4° C, in the Servall preparative centrifuge, except where indicated. The resulting tan colored supernatant was used immediately, in the experiments. An attempt was made to maintain the liver preparation at a temperature of 0-4° C, during the entire operation described above.

The measurement of glucose in each flask was performed using glucose oxidase of the highest available purity obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Initially, protein free filtrates were prepared according to the Nelson-Somogyi method. However, since the additional pipetting involved in preparing the filtrates introduced a possible source of error, an alternate method was searched for. It was reasoned that if a small enough quantity of the contents of

each flask was added directly, at the appropriate time, to a large enough quantity of glucose oxidase solution any inhibitory agents present might be sufficiently diluted out to ensure at least the same degree of accuracy as that entailed in the procedure employing the preparation of filtrates. It was found, that 0.1 ml of the contents of a flask added to 5.0 ml of glucose oxidase reagent gave almost identical results to those in which filtrates were prepared. Therefore, the time consuming step of preparing filtrates was eliminated. The results of a standard curve indicating linearity is presented in the appendix. Since biotin was present in different concentrations in each flask in some experiments, it was necessary to exclude the possibility that biotin might inhibit or stimulate the enzyme glucose oxidase which in turn would give apparent glucose utilization rates by the liver preparation which were in error in proportion to the inhibitory or stimulatory effect. Biotin in concentrations ranging up to ten times those used in the experiments had no effect on the measurement of glucose in a standard glucose solution using glucose oxidase.

The pH of the incubation media, measured using a Beckman pH meter, did not vary more than 0.15 pH units throughout the course of an experiment.

CHAPTER IV

DATA

A. Experiments Involving Crystalline Yeast Hexokinase

Since early literature reports indicated a role of biotin in some initial stage of yeast glucose utilization, the possibility exists that biotin is a cofactor in the hexokinase reaction. To test this hypothesis, crystalline yeast hexokinase of the highest commercial quality was obtained. This enzyme catalyzes the ATP (Mg^{+2}) dependent phosphorylation of glucose and to a lesser degree other hexoses, according to the following reaction:

D-glucose + ATP (Mg^{+2}) $\longrightarrow$ D-glucose-6-phosphate + ADP + H^+

The assay method employed in this study is that of Colowick (46). The principle of the method is that in the hydrolysis of ATP, H^+ is produced in stoichiometric proportions to the amount of glucose phosphorylated and therefore measurement of the amount of H^+ produced per unit time is an indication of the velocity of the enzyme-catalyzed reaction. If a colored indicator, with a pKa which is identical to that of the

buffer employed, is added to the system, the hydrogen ion produced in the reaction will cause a change in the buffer from salt to acid form. This change will be accompanied by an identical change in the indicator forms, resulting in a change in the color of the system which in turn can be measured as a change in optical density at the appropriate wavelength. The change in optical density can be correlated with the amount of hydrogen ion produced by titration and therefore with the amount of glucose phosphorylated.

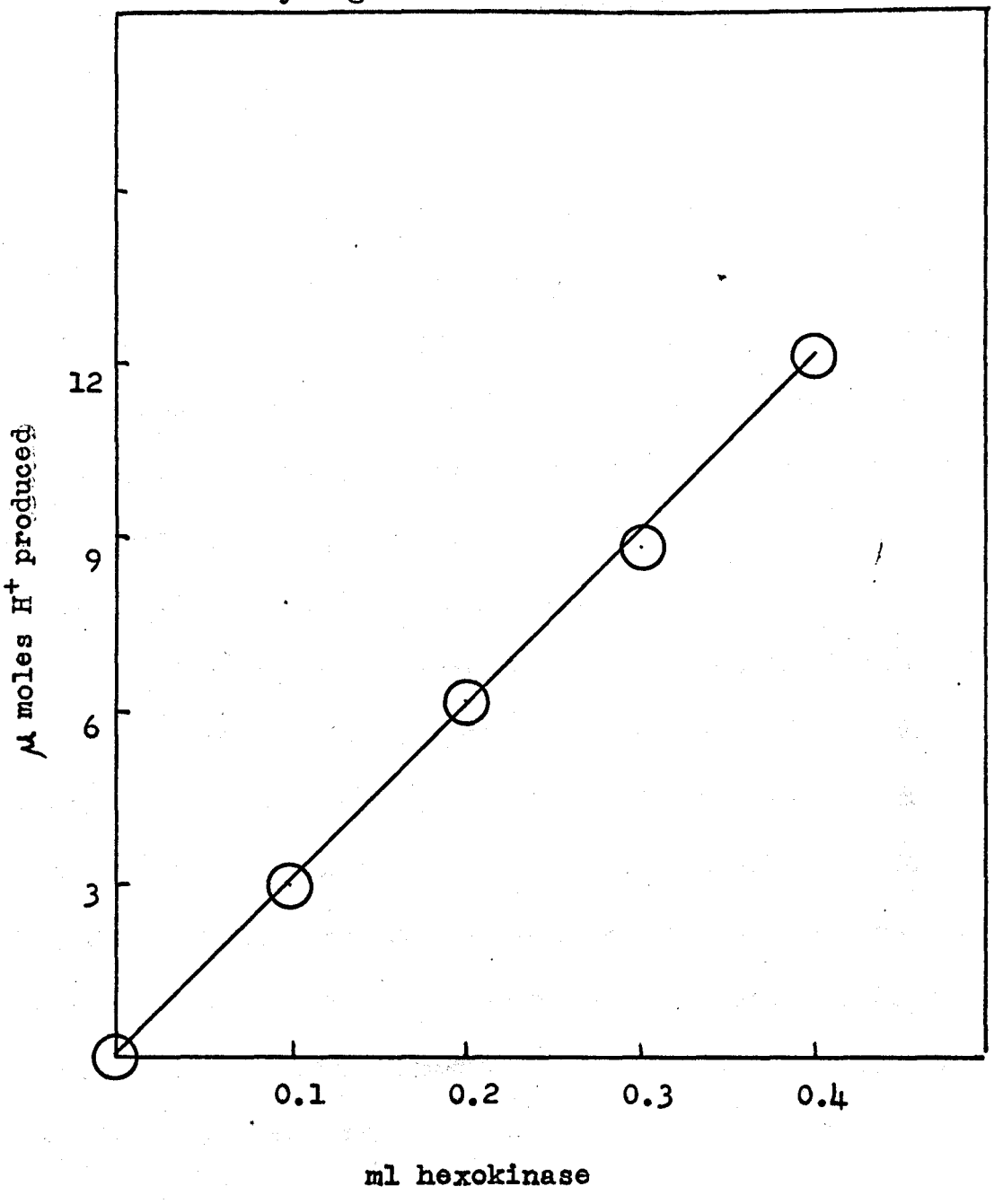
The fundamental objective in these experiments was to determine whether or not avidin, which specifically inhibits all known bound biotin enzymes, would inhibit the phosphorylation of glucose by yeast hexokinase. In the experiments to be described in the following paragraphs the system employed is indicated in the legend under the appropriate graph or table.

1. Effect of the Concentration of Glucose, ATP (Mg), and Hexokinase

This experiment is designed to establish the validity of the assay method. From Graph I it can be seen that the rate of production of hydrogen ion is directly proportional to the concentration of hexokinase. One-tenth of hexokinase solution, with an estimated activity of 32 units/

Graph I.

Effect of the Concentration of Hexokinase on The Rate of Production of Hydrogen Ion



ml or 32 μmol glucose phosphorylated/min/mg protein/ml, was used. The optimal concentrations of ATP (Mg) and glucose are indicated also in Graph II and Graph III and were also used in all further experiments involving yeast hexokinase.

2. Effects of Dialysis

This experiment was designed to eliminate the possibility that a dialyzable cofactor was necessary for activity of the hexokinase preparation. As can be seen from Table I, extensive dialysis resulted in no loss of total activity of the hexokinase enzyme. Therefore, if a cofactor is necessary, the conclusion is reached that it is in a form which is non-dialyzable.

3. Effect of the Concentration of Biotin

While the negative results of dialysis eliminated the possibility of biotin being a dialyzable cofactor, the possibility existed that added biotin might in some manner alter the activity of the enzyme in a manner similar to the activation of acetyl-CoA carboxylase by isocitrate. To test this hypothesis, crystalline biotin dissolved in doubly distilled water was added in the indicated concentrations, as shown in Table II. As can be seen, free biotin caused no change in enzyme activity with the concentrations of biotin used.

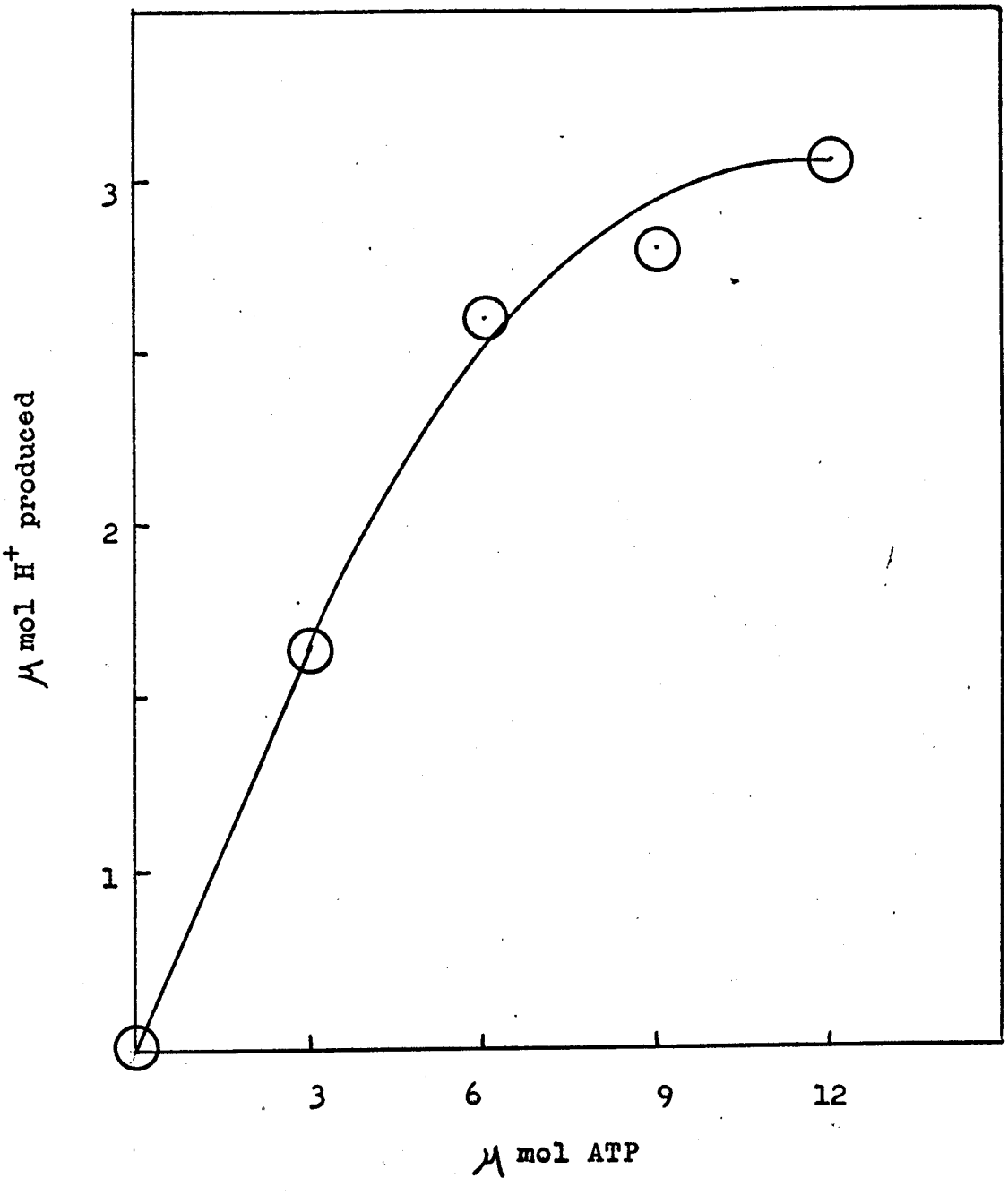
TABLE I

	Hours Dialysis				
	0	1.0	2.0	3.0	8.0
Activity μmol H^+/min	2.9	3.0	3.1	2.8	3.0

The complete system is the same as that in Graph I except that 0.1 of hexokinase was used. Dialysis was carried out against 0.05 M glycyl-glycine buffer, pH 8.6, for the indicated lengths of time.

Graph II.

Effect of the Concentration of ATP on the Hexokinase Reaction



Graph III.

Effect of the Concentration of Glucose on the Hexoinase Reaction

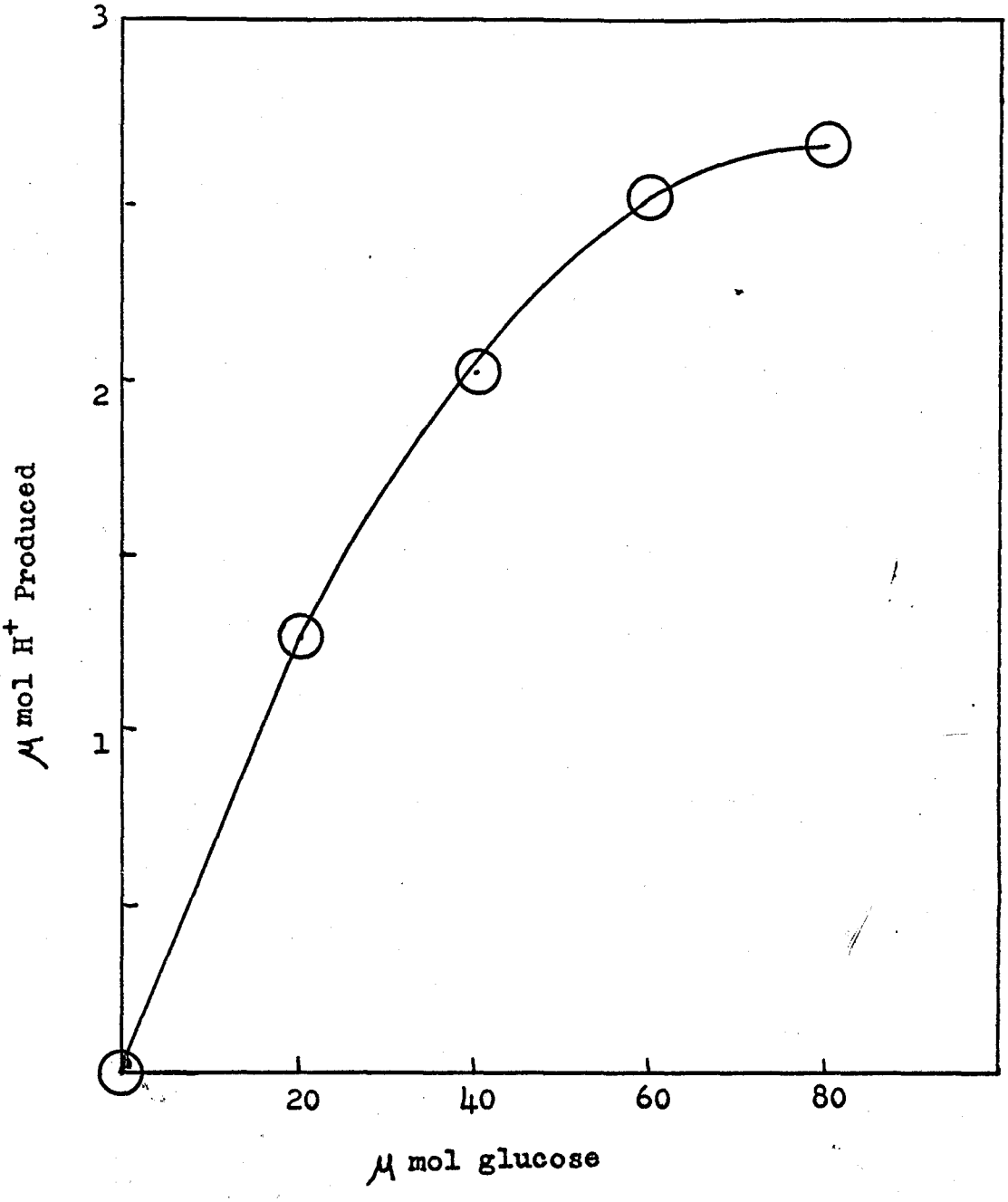


TABLE II

	Concentration of Biotin μmol/ml				
	0	4	8	12	16
Activity μmol H^+/min	3.0	3.2	3.0	3.1	2.9

The conditions of the experiment are exactly the same as those in Table II, with the indicated amounts of biotin added.

4. Effect of Avidin

As has been stated earlier, avidin has been used extensively as a specific inhibitor of enzymes containing bound biotin as an active cofactor. The curves in Graph IV, represent the addition of increasing amounts of avidin to the hexokinase preparation, preincubation for 30 minutes at 0° C and initiation of the reaction with the addition of Mg (ATP). While the curves appear to indicate inhibition, it must be remembered that avidin is a basic protein and at the pH of incubation will have the effect of increasing the buffer capacity of the system and therefore decreasing the apparent rate of production of hydrogen ion. A careful analysis of the data, (Table III) shows that the initial rates in all cases are not statistically different. From this experiment, it is concluded that biotin is not a cofactor in the hexokinase reaction, at least in a form which is combinable with avidin.

B. Whole Animal Experiments

The negative results obtained with crystalline yeast hexokinase do not exclude the possibility that biotin is somehow involved in glucose utilization of the intact cell, disrupted cells, or the whole organism. In order to test the latter possibility, biotin was injected into male

Graph IV.

The effect of the Concentration of Avidin on The Hexokinase Reaction

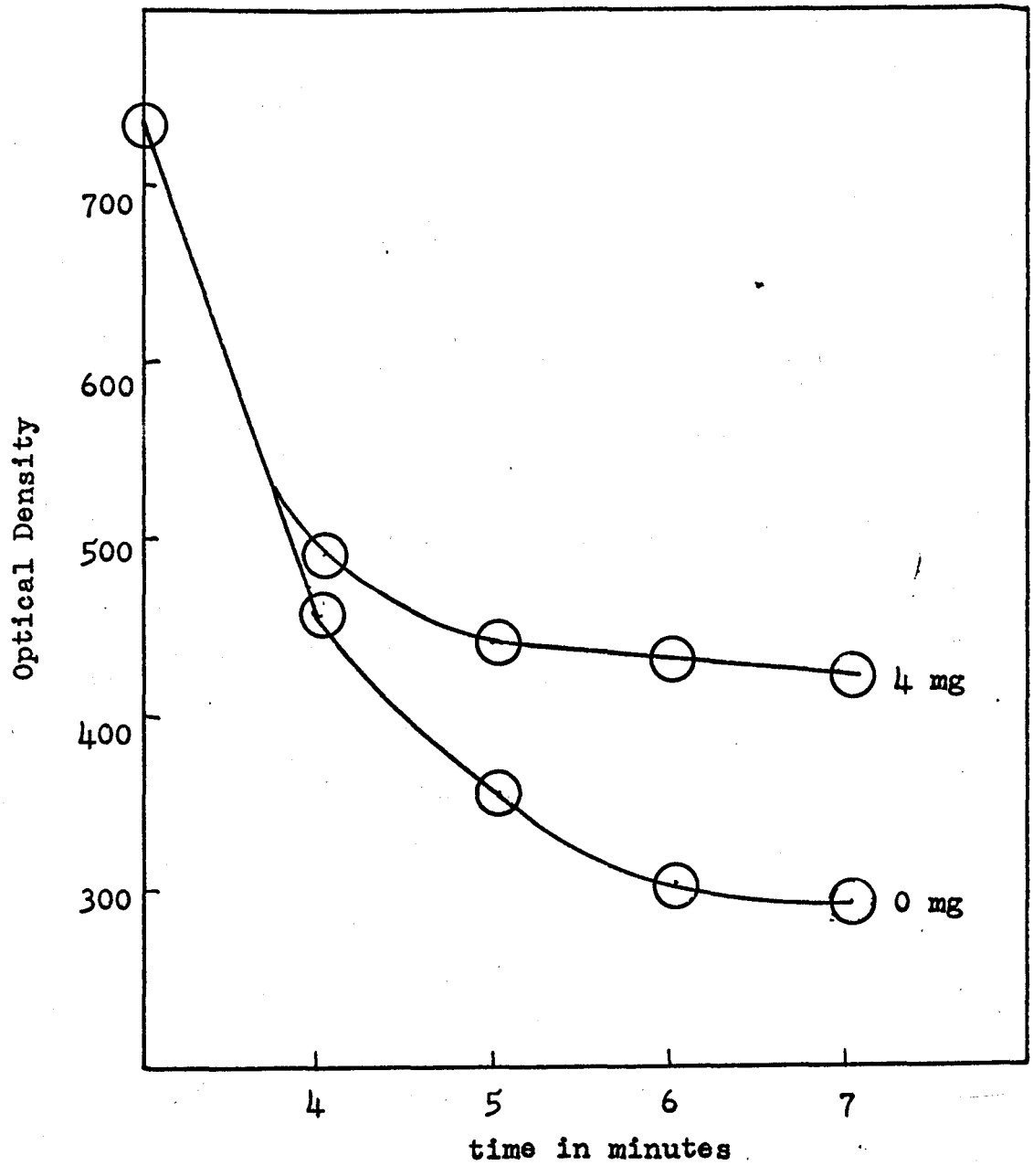


TABLE III

	mg Avidin				
	0	1	2	3	4
Activity μmol H^+/min	2.7	2.9	2.7	2.7	3.0

The conditions of the experiment are exactly the same as in Table II, except that in the control flask, NaCl was added in equivalent amounts to those needed to solubilize the avidin. (1% NaCl)

albino rats and at the indicated times blood glucose was measured using glucose oxidase which is specific for β-D-glucose (47). The exact methods employed in using glucose oxidase are given in the appendix. The conditions of the experiment are indicated in the legend in Graph V. As can be seen, biotin produced a small but consistent hypoglycemic effect in treated rats compared to the saline controls. However, the identical experiment repeated at a later date gave results which were essentially negative when compared to the previous experiment. Fortuitously, it was recalled that the animals in the first experiment were not in a fasting condition, while the animals in the experiment of the later date had inadvertently not been fed for approximately 36 hours. Therefore, the effect of fasting on the hypoglycemic effect of biotin was studied. The results are given in Table IV. As can be seen, rats fasted for 24 hours do not respond to injection of biotin while the greatest effect is observed after feeding 4-6 hours before the injections. The mechanism of this phenomenon remains obscure. However, as mentioned in an earlier paragraph, many enzymes have recently been found to be quite sensitive to the effects of fasting. Because of this, it was decided to study the effects of biotin on <u>in vitro</u> glucose utilization. Liver was chosen for the studies because of the presence of glucokinase, the low K_m enzyme which

Graph V. The Effect of the Injection of 30 μg, Biotin into male albino Rats

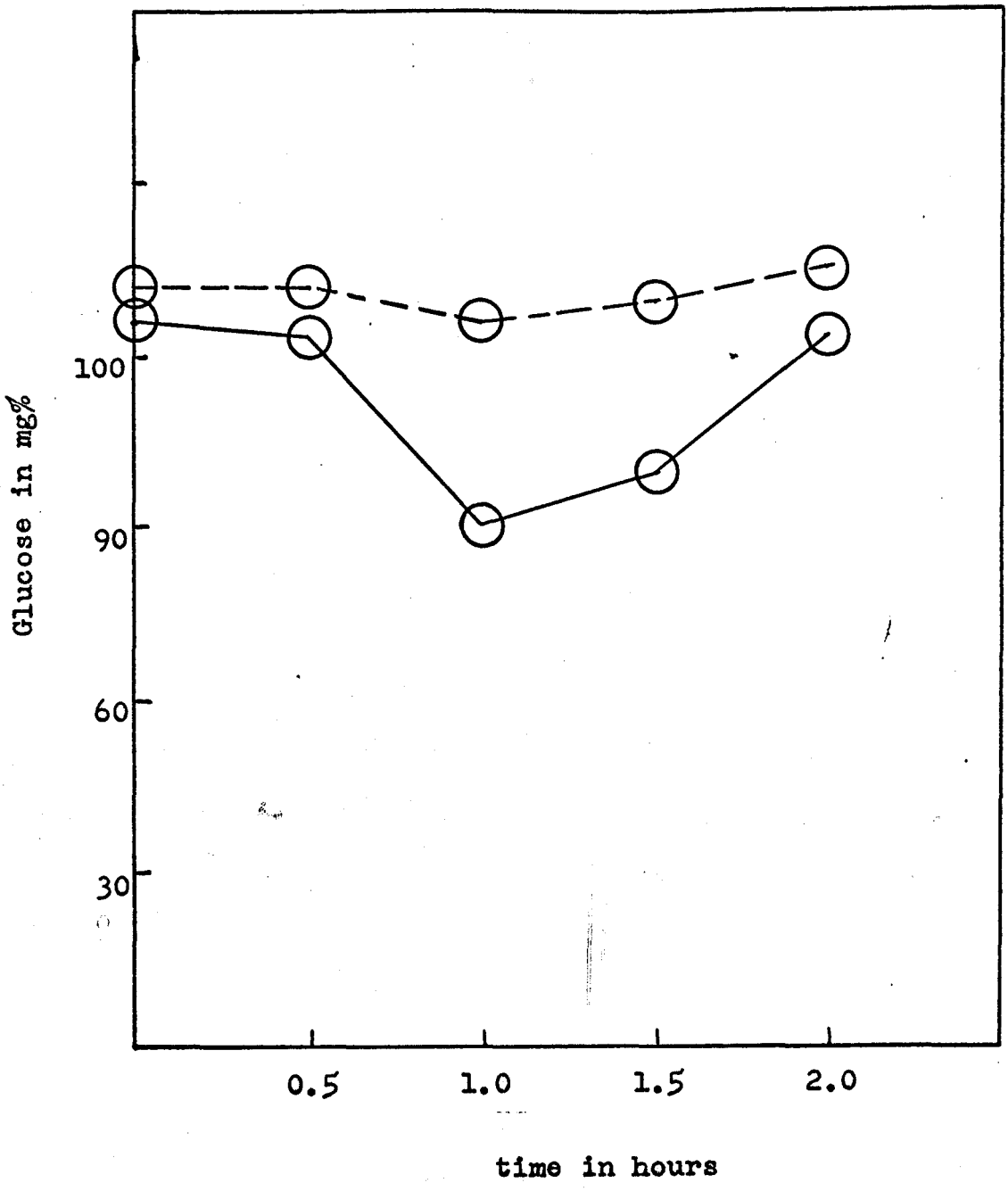


TABLE IV

CONDITION OF ANIMAL

	Fed	Fasted 24 Hours	Fed 5 Hours	Fed 12 Hours
Glucose % Control	82 ± 7%	102 ± 5%	80 ± 8%	79 ± 8%

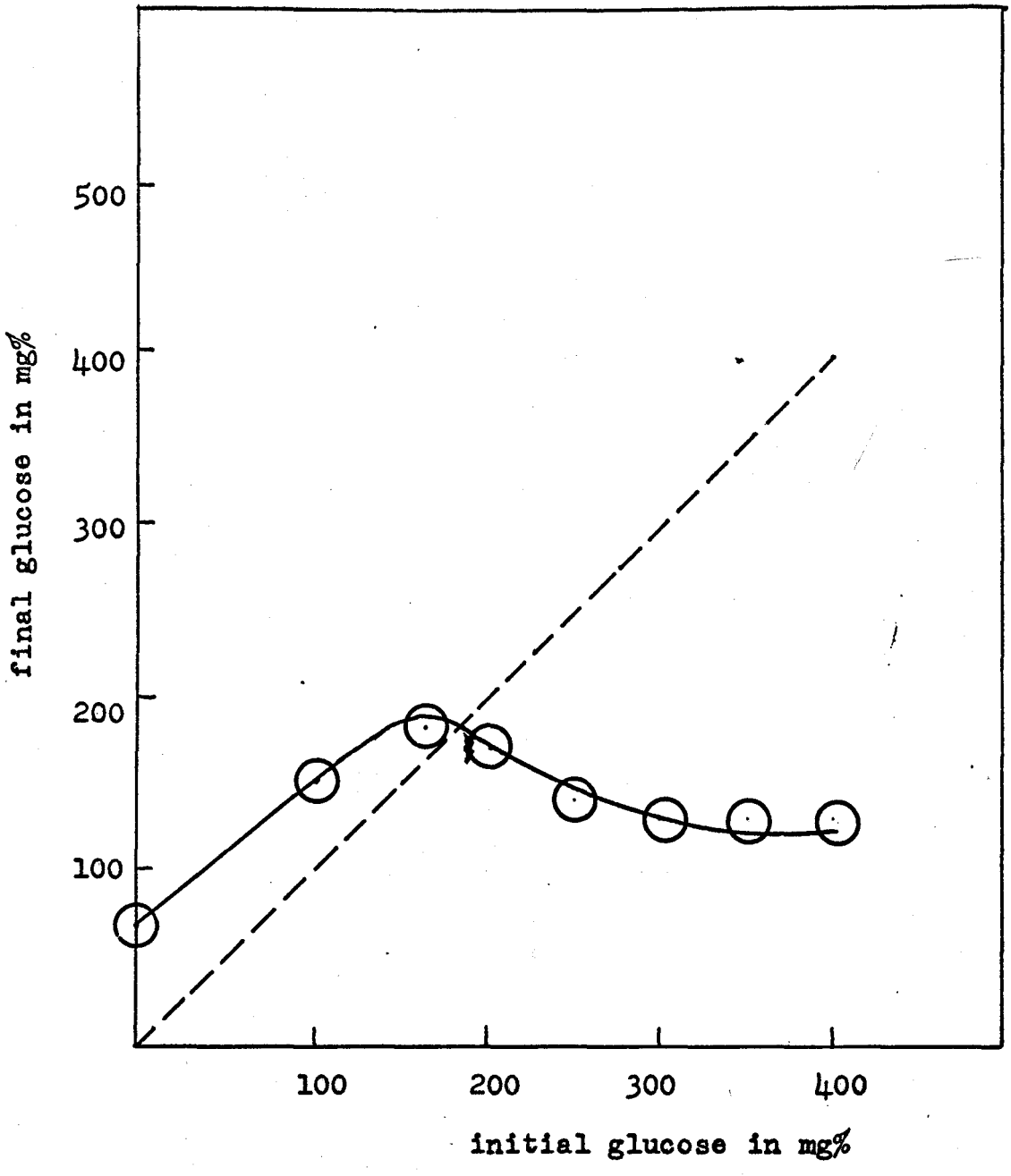
The experimental conditions are exactly the same as those described in Graph VI. The results are calculated from data similar to that obtained in Graph VI, and are expressed as per cent of the control level of glucose one-half hour after injection of biotin.

specifically phosphorylates β-D-glucose and is markedly responsive to diet, as mentioned earlier.

C. Liver Homogenate Studies

It was recognized that liver is the main source of blood glucose and therefore liver homogenates containing glycogen might be expected to both utilize glucose and produce it by hydrolysis of glycogen. Since the liver is known to store glycogen post-prandially when the portal vein concentration of glucose is high, it was decided, as an initial experiment, to measure the glucose found after incubation as a function of the initial glucose present in the media. The results are given in Graph VII. As can be seen from the point where the curve crosses the 45 degree dotted line, the preparation was producing glucose until the initial glucose concentration was somewhat greater than 150 mg %. The glucose level for the remainder of the experiment was chosen to be 300 mg %, since glucose utilization was the desired parameter to be measured. The exact conditions of the experiments are given in the legends in the tables and graphs.

Graph VII. The Effect of the Initial Concentration of Glucose verses the Final Concentration of Glucose



1. The Effect of Fasting on Liver Homogenate Glucose Utilization

With the results of injection of biotin into intact rats in mind, the effects of a previous fast before sacrifice, on homogenate glucose utilization, was studied. The results are given in Table V. As can be seen from the data, a 24 hour fast has virtually no effect on the homogenate glucose utilization. However, a 48 hour fast diminishes glucose utilization by the liver preparation by 75% compared to control levels. Re-feeding the fasted animals as little as 8 hours before sacrifice restored levels of glucose utilization to almost those of normal fed controls. Except where indicated, all further experiments were performed on rats which had been fed ad libitum about four hours before sacrifice and had at no time been in a prolonged fasting condition.

2. The Effect of Various Parameters on Liver Homogenate Glucose Utilization

a. The effect of duration of incubation.

In order to minimize the length of an experiment and also to attempt to measure initial rates of utilization, the effect of the duration of incubation was studied. The results are given in Graph VIII. As can be seen, while glucose utilization still proceeds after a 15 minute

TABLE V

EFFECT OF FASTING ON HOMOGENATE GLUCOSE UTILIZED

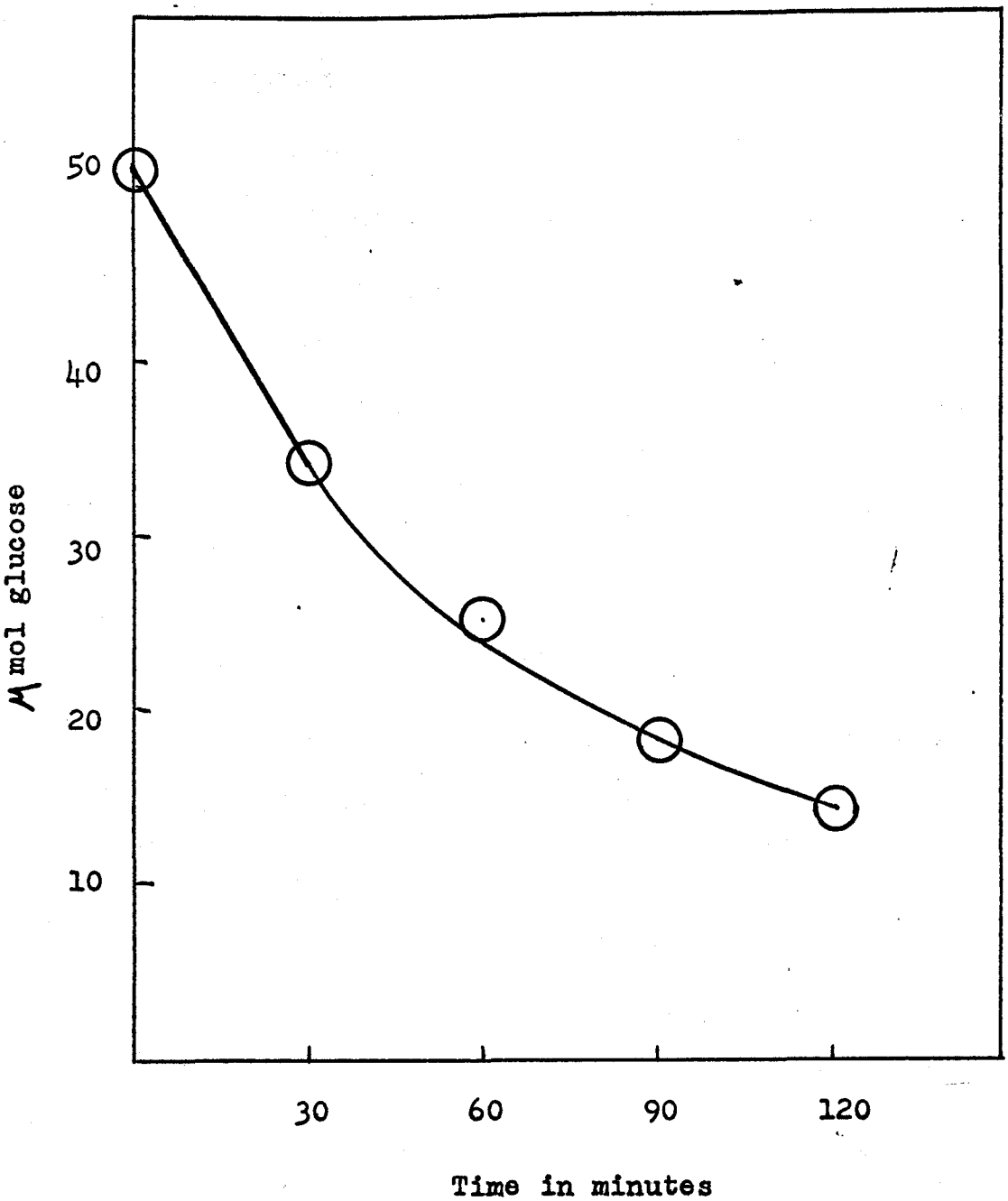
Glucose Utilization μmol/min/gm

Fed	Fasted 24 Hours	Fasted 48 Hours	Fed 8 Hours
5.5 ± 0.5	5.3 ± 0.4	1.2 ± 0.5	4.5 ± 0.6

The conditions of the experiment are the same as those described in Graph VII, except for the indicated condition of the animal. The results are the average of three rats with the standard deviation indicated.

Graph VIII.

The Effect of Time on Glucose Utilization by the Rat Liver Homogenate



incubation period, the greatest amount of glucose disappeared from the medium early in the experiment. For this reason, the length of incubation was minimized to 15 minutes with measurements being made at 0 time, 5 minutes, 10 minutes, and 15 minutes.

b. The effect of the concentrations of ATP, NAD, and glutathione on glucose utilization.

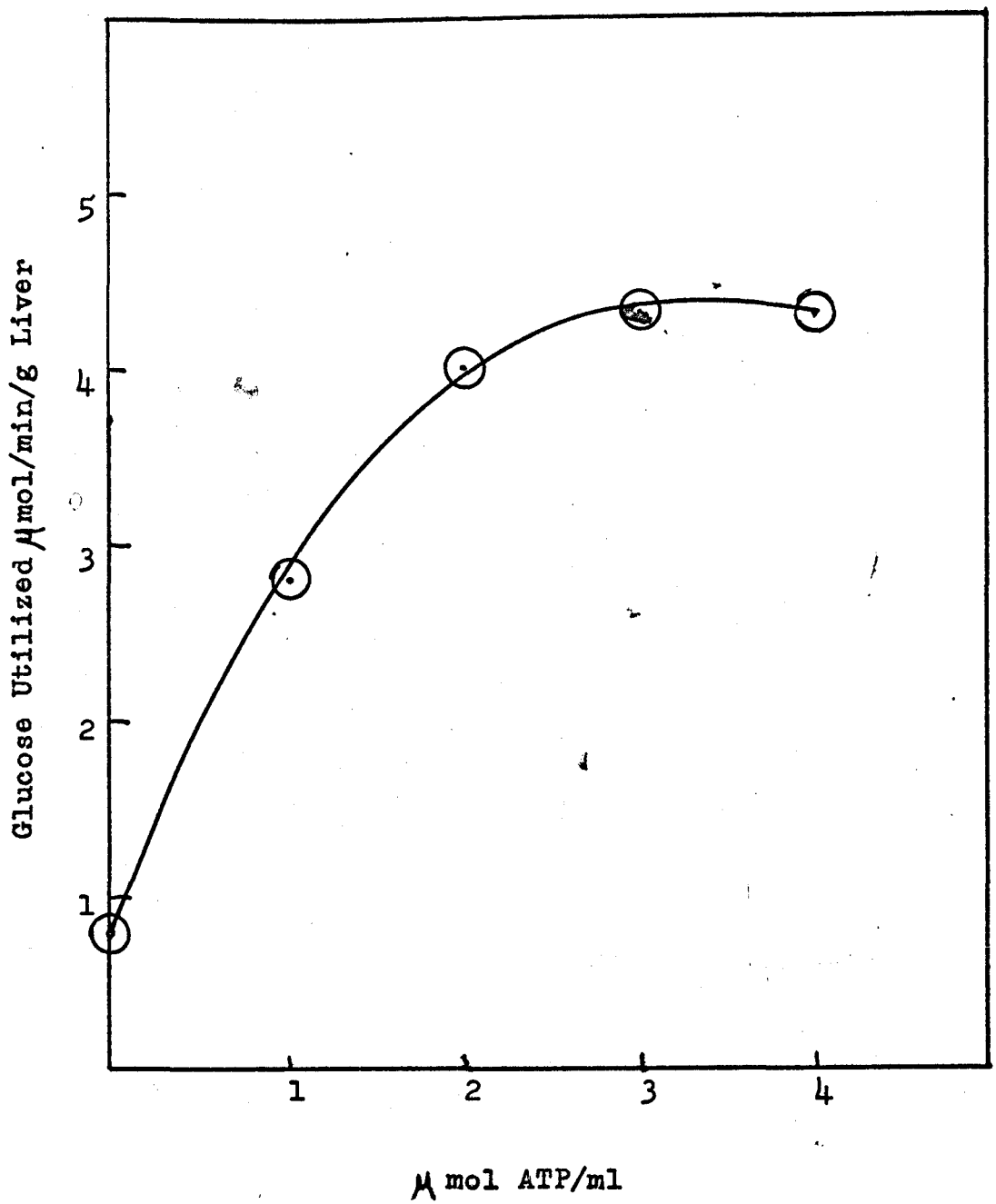
The results of these experiments are given in Graphs IX, X, and XI. The system had an almost absolute requirement for ATP, and was substantially stimulated by the addition of NAD and glutathione. The maximal concentrations of the various substitutes were used in the remaining experiments.

c. The effect of biotin on glucose utilization.

The results of increasing concentrations of biotin are given in Graph XII. As noted in the earlier experiments there is substantial utilization of glucose without added biotin. However, the initial rate of glucose utilization is stimulated up to 40% with the addition of 0.024 mol of biotin to the system. Furthermore, with increasing concentrations of biotin, the effect of lesser concentrations is inhibited and utilization below control levels is found at 0.072 mol biotin. As stated earlier, the liver homogenate used was centrifuged at 15,000 x G for 20 minutes to

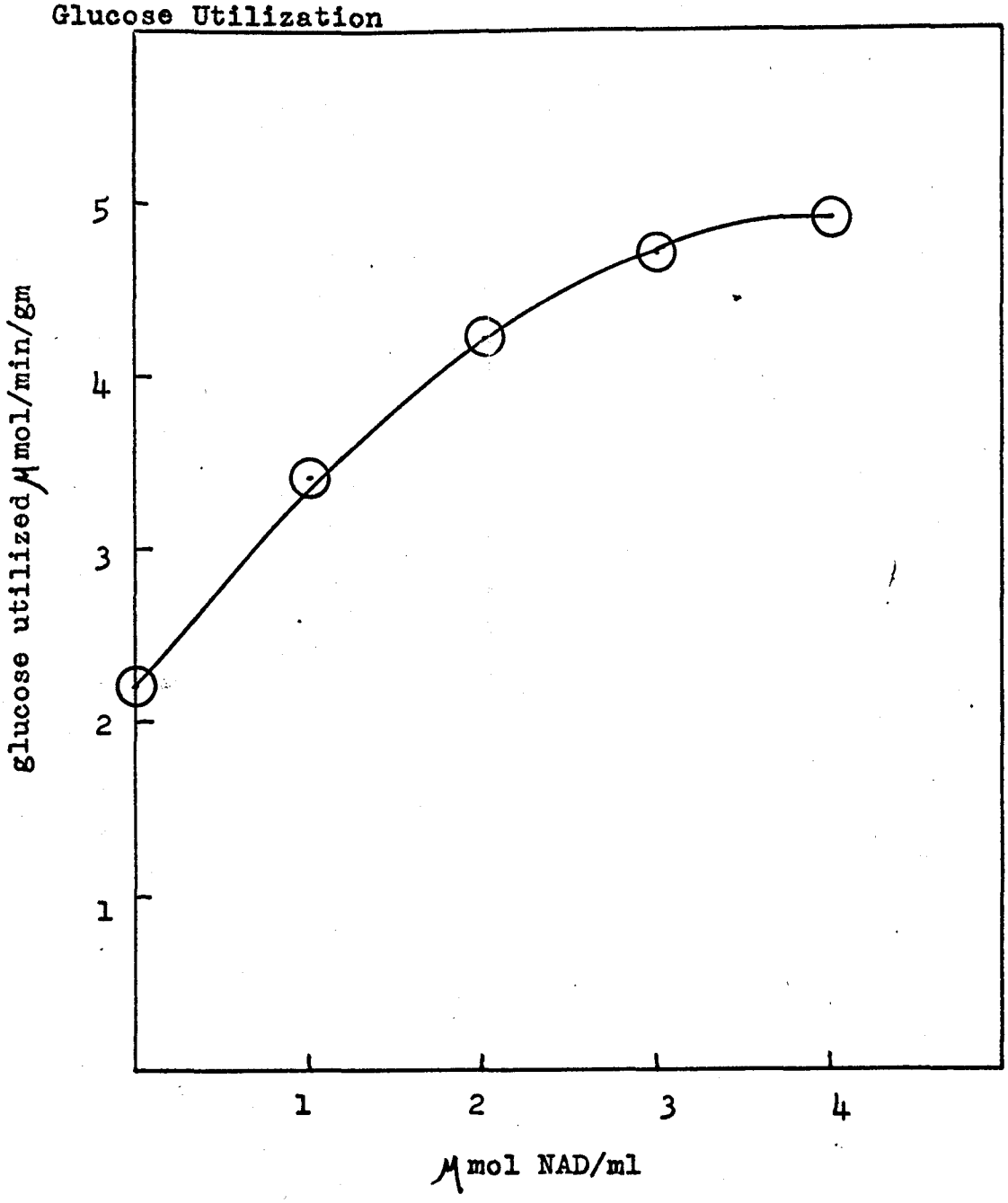
Graph IX.

The Effect of the Concentration of ATP on Liver Homogenate Glucose Utilization



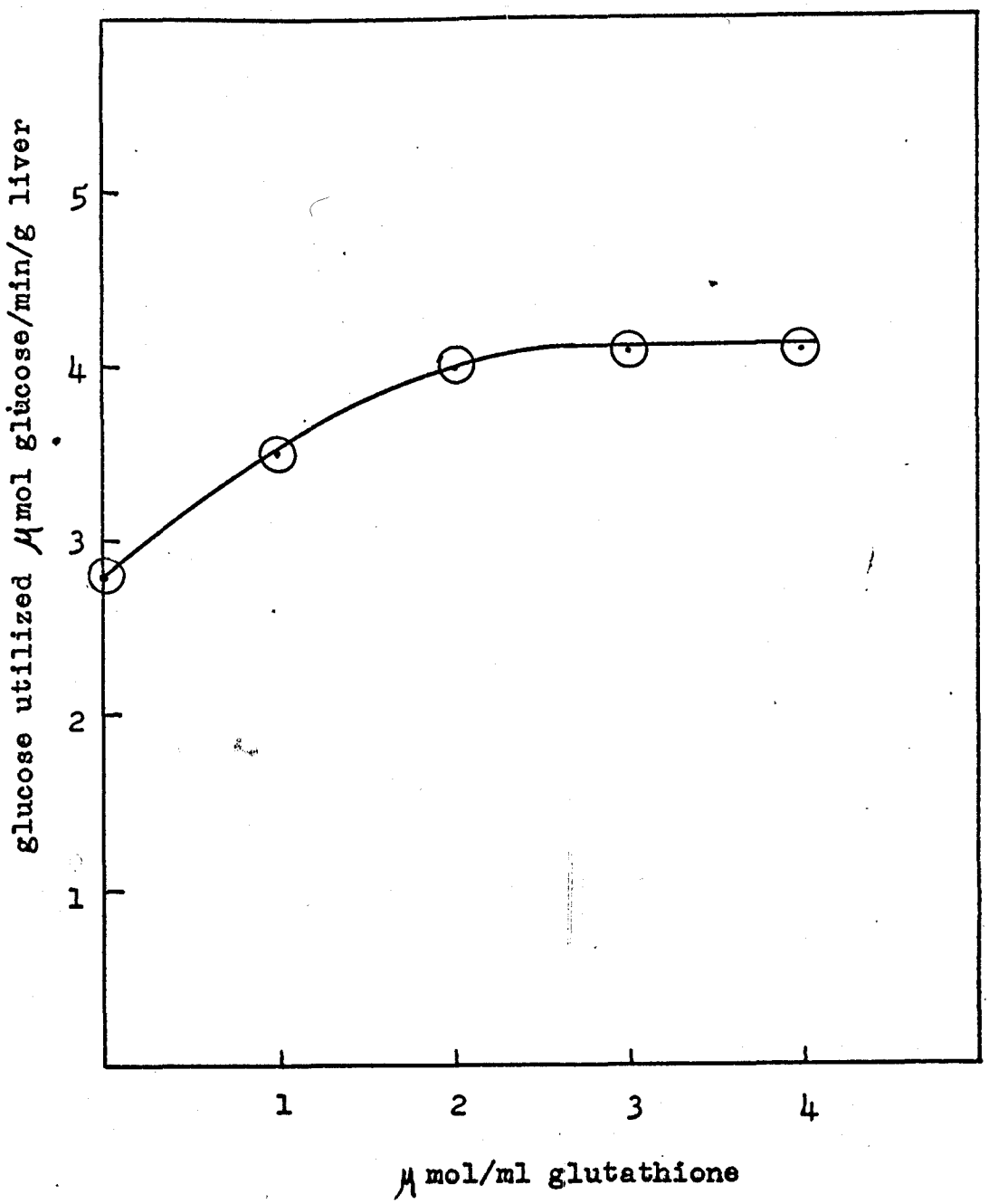
Graph X.

The Effect of the Concentration of NAD on Liver Homogenate Glucose Utilization



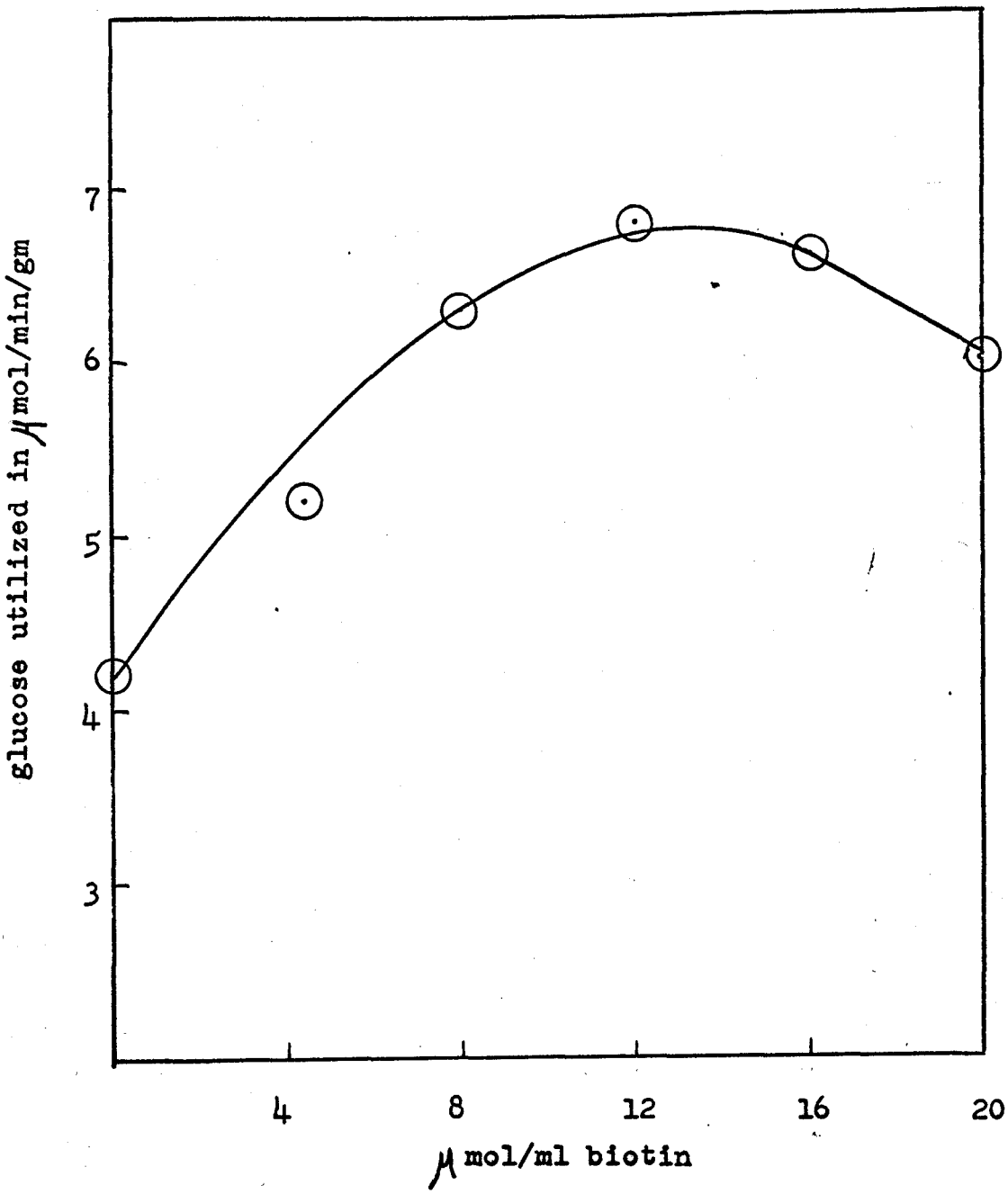
Graph XI.

The Effect of the Concentration of Glutathione on Liver Homogenate Glucose Utilization



Graph XII.

The Effect of the Concentration of Biotin on Liver Homogenate Dlucose Utilization



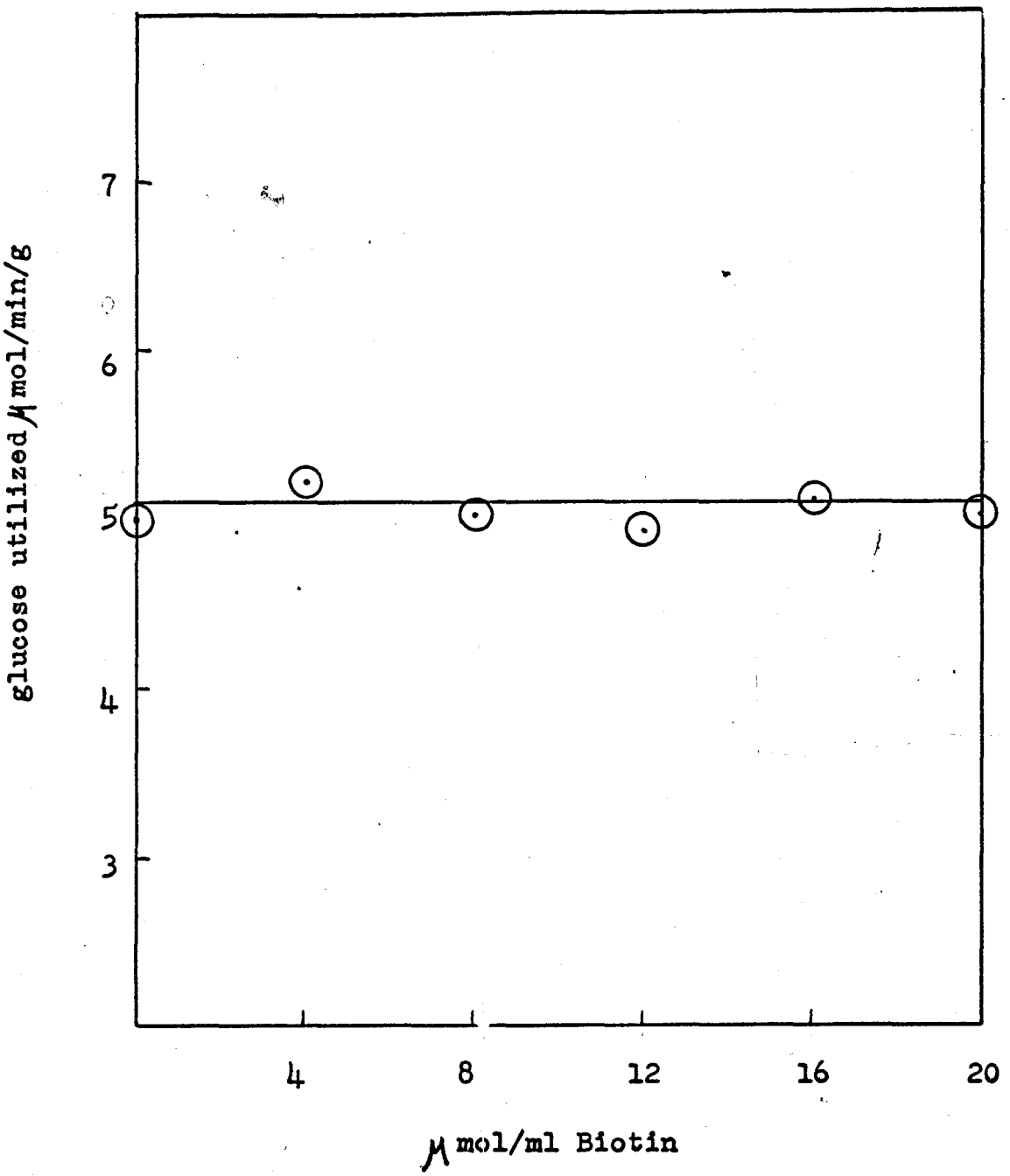
remove mitochondria and therefore Kreb cycle function. The resulting system theoretically contained cell sap with all soluble material plus all particulate matter lighter than mitochondria. The most notable elements of this particulate matter are the microsomes and other fragments of the endoplasmic reticulum which have been strongly implicated in amino acid activation and protein synthesis. Since glucokinase is found exclusively in the soluble portion of liver homogenate, it seemed desirable to determine whether or not the observed effect of biotin was due primarily to an effect on the soluble enzyme portion.

d. The effect of centrifugation at 100,000 x G for 30 minutes.

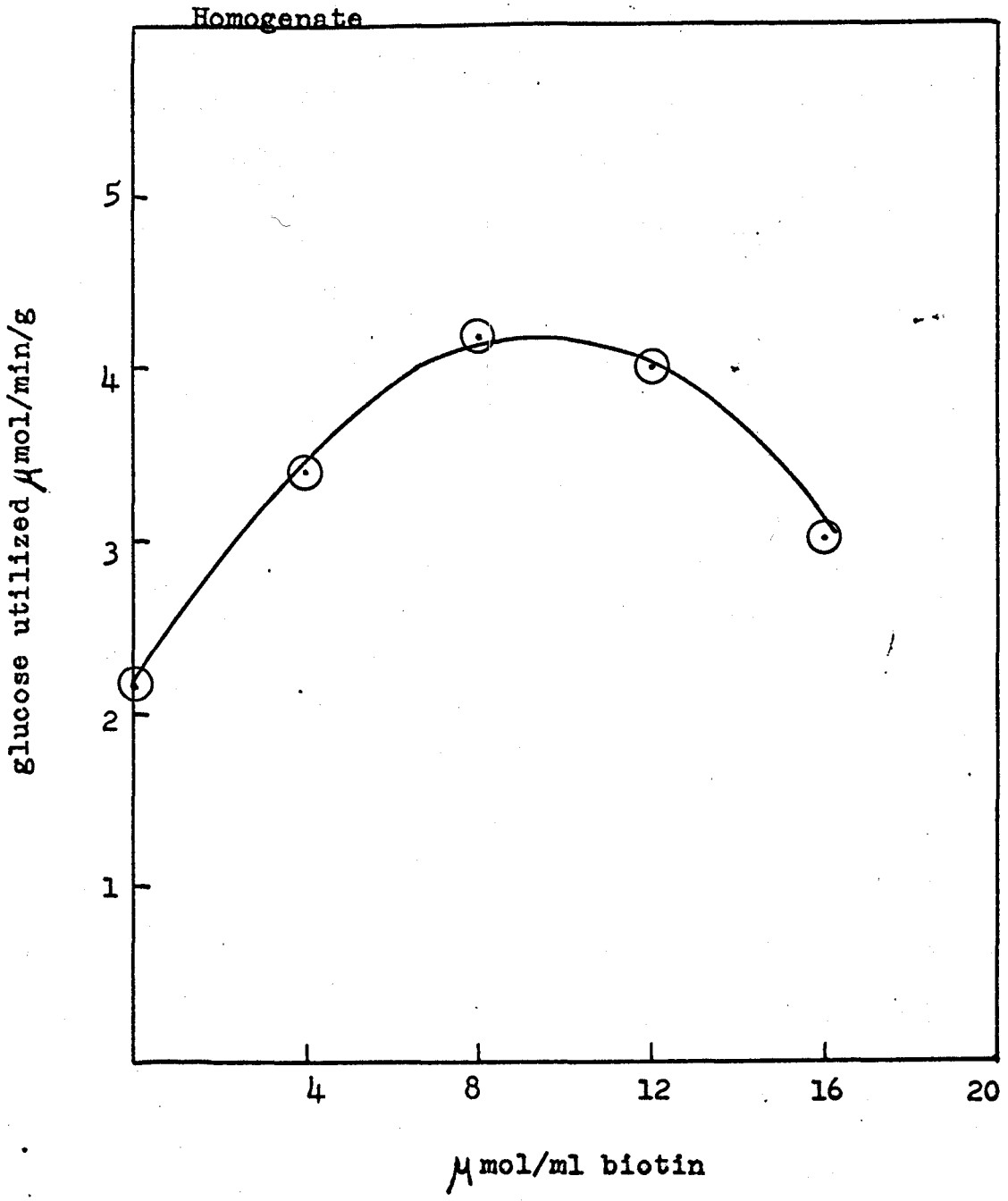
Most, if not all, of the particulate matter is removed upon centrifugation at the above force and time. The resulting supernatant was used as a source of enzyme and glucose utilization measured. The results are reported in Graph XIII. Interestingly, while glucose utilization proceeded at a significant rate compared to controls, the addition of biotin had no effect on glucose utilization at any of the concentrations of biotin used. However, as shown in Graph XIV, recombination of the sediment obtained from centrifugation with the supernatant resulted in a preparation which responded to the addition of biotin in a manner similar to

Graph XIII.

The Effect of Biotin on The Glucose Utilization of the 100,000 x G Liver Supernatant



Graph XIV. The Effect of Biotin on The Reconstituted Liver Homogenate



that reported earlier. Therefore, we must conclude, that particulate matter is necessary for the effect of biotin to be shown and that addition of biotin has no effect on the soluble enzymes alone. This particulate matter is tentatively assumed to be microsomal in nature.

e. The effect of preincubation of the liver preparation with biotin.

Since the effect of biotin on glucose utilization is most probably caused by an effect of biotin on the liver preparation, various concentrations of biotin were preincubated with the liver homogenate and the reaction initiated by addition of substrate. The initial experiment was performed with the addition of biotin to a level of 0.008 μmol/ml which is the optimal concentration of biotin in regards to glucose utilization in previous experiments. The results are given in Table VI. The data show almost exactly the opposite results obtained in previous experiments when this concentration of biotin is preincubated with the liver preparation. It will be recalled that higher concentrations of biotin inhibited the effects of lower concentrations on glucose utilization. With this in mind, the effect of concentration of biotin preincubated with liver homogenate was studied. The results are given in Graph XV. The data show that the stimulatory effect of biotin is observed with much lower concentrations

TABLE VI

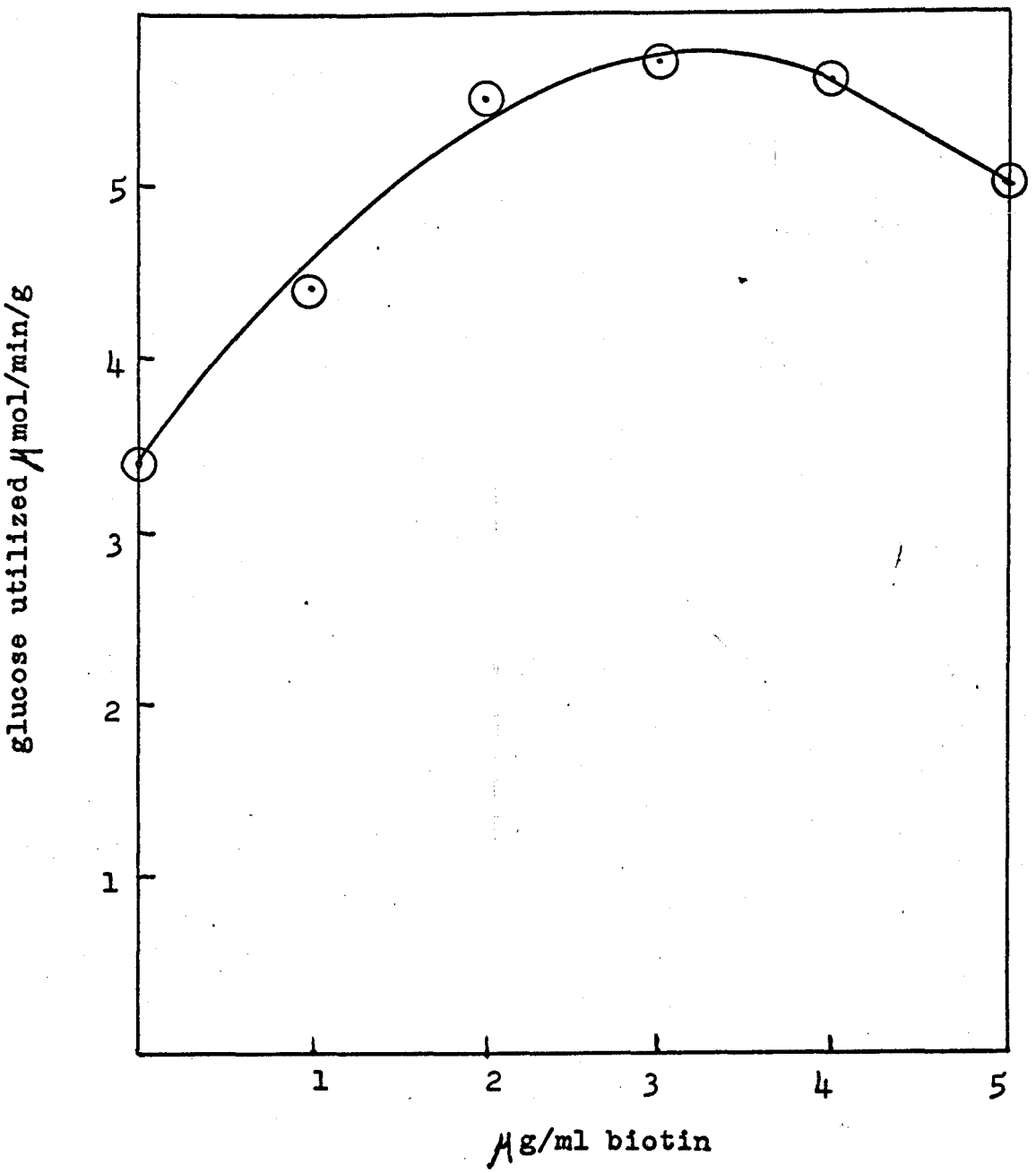
PREINCUBATION OF BIOTIN WITH LIVER HOMOGENATE

	Glucose Utilization (μmol/min/gm)
Control Utilization	4.7 ± 0.4
Biotin Preincubated	3.1 ± 0.5
Biotin Added	6.2 ± 0.5

The conditions of the experiment are exactly the same as those described in Graph XII. The amount of biotin added was 2 μmol/ml with respect to the total volume in each flask after the reaction was initiated.

Graph XV.

The Effect of the Concentration of Biotin Preincubated with Liver Homogenate



The complete system is the same as that described in graph XII, except for the indicated changes in biotin concentration.

of biotin than in previous experiments. Higher concentrations still inhibit the effects of lower concentrations.

f. The effect of preincubation of avidin with the liver preparation.

In analogy with the yeast hexokinase experiments, avidin was tested as an inhibitory agent on glucose utilization by the liver preparation. As can be seen in Graph XVI, avidin was without effect on glucose utilization in those systems to which no biotin had been added. Moreover, in those flasks which contained biotin preincubated with added avidin, the effect of biotin was not abolished; however, in those systems which contain preincubated avidin and added biotin, the effect of biotin was almost completely abolished. This experiment also appears to preclude the possibility that biotin-mediated carboxylation reactions in the soluble cell sap play any measurable role in some initial state of glucose utilization since all biotin enzymes reported to date are completely inhibited by excess avidin.

CHAPTER V

DISCUSSION

Biotin has heretofore not been implicated in playing a role in mammalian glucose utilization. While some authors, as noted earlier, have reported signs of abnormal carbohydrate metabolism in biotin deficiency, an effect of free biotin on mammalian glucose uptake has not been reported previously in the literature. For this reason, the data must be carefully examined. The initial assumption, that biotin might be a co-factor, bound or free in some initial stage of glucose utilization, has not been entirely substantiated or disproved. While it is possible, that avidin may not bind irreversibly all forms of biotin, the form which it does not bind has not been shown to exist. Moreover, all known biotin enzymes are drastically inhibited by avidin. In experiment 2f, avidin added in excess amounts failed to inhibit glucose utilization with no added biotin present which seemed to indicate that those enzymes involved in biotin carboxylation reactions do not play a measureable role in initial glucose utilization of the present system. Also, the possibility that free biotin

existing in the soluble cell sap is involved in glucose utilization appears doubtful since avidin did not inhibit glucose uptake by the preparation. However, biotin added to the system in extremely small quantities, 4×10^{-6} M, did exert a rather significant stimulatory effect.

The fact that this stimulatory effect required the presence of particles which sediment between 10,000 x G and 100,000 x G is interesting. The main constituent of these particles are those involving amino acid activation, transfer, and protein synthesis in general. The distinct possibility exists that the effect of biotin is due to an effect occurring inside these particles and one therefore which is inaccessible to avidin due to the probable impermeability of these particles to such a large protein molecule. The truth of this statement remains to be proven, but is supported by the experiment in which preincubation of the liver homogenate with biotin before addition of avidin resulted in a stimulatory effect.

The in vivo experiments are also of interest. However, it is extremely doubtful whether the observed hypoglycemic effect of biotin in rats is physiological. The amount injected was 30 μg and the immediate volume of distribution could be no more than 20 ml. If we assume a maximum initial concentration of 1.0 μg% then the final concentration of biotin would be 200 μg%, or about 200 times the normal. However, in

vivo hypoglycemia effects coupled with the results of the in vitro data does lend some credence to the possibility that biotin may participate directly in mammalian glucose utilization. As noted earlier, 4 x 10 M biotin is not far from the reported normal concentration of this vitamin in whole liver.

A few words should be said about the observed rates of glucose utilization in vitro compared with the probable resting post-prandial removal of glucose from the extracellular space in vitro. The data show (with no added biotin) an initial rate of glucose disappearance of about 4.0 mol glucose/min/gm liver. This is equivalent to 0.720 mg glucose/min/gm liver. If we assume a liver weight of 10.0 g, then 3.60 mg of glucose can be utilized per minute. An extracellular space of 20.0 ml coupled with a glucose concentration of 1000 mg% (1) gives a total amount of extracellular glucose of 200 mg. If we were to drop the glucose concentration to 100 mg%, we would have to remove 180 mg of glucose which would take about 25 minutes according to the data. This rate then would appear to be at least representative of in vitro glucose disappearance.

As was stated in the initial section of this thesis, biotin was postulated to play a role in mammalian glucose utilization on the basis of the known biochemical pathways coupled with the theoretical effects of

removal of biotin enzymes from the metabolic disorder, diabetes mellitus. It is fully realized that the data presented in this thesis does not prove or disprove the postulate that biotin and diabetes mellitus are causally related. However, the data compiled here plus the literature available do not eliminate the postulate from the realm of possibility.

CHAPTER VI

CONCLUSIONS

The vitamin d-biotin was postulated to play a role in some initial stage of glucose utilization. The conclusions reached from the data obtained are:

1. Biotin is probably not an active cofactor in crystalline yeast hexokinase.

2. Biotin is probably not an active cofactor in any of the soluble rat liver enzymes involved in the glycolytic pathway.

3. Biotin mediated carboxylation reactions probably play no role in the initial stages of glucose utilization.

4. Free biotin does exert a pronounced effect on initial glucose utilization only in the presence of the particulate matter which sediments between 10, 000 G and 100, 000 x G.

5. The effect of free biotin on glucose utilization is possibly due to an effect exerted within the particulate matter and therefore in a form which cannot combine with avidin.

APPENDIX

A. Abbreviations Used

NAD, Nicotinamide-adenine dinucleotide

ATP, Adenosine Triphosphate

CoA, Coenzyme A

B. Glucose Oxidase Calibration Curve

The method employed in measurement of glucose proved linear up to 600 mg%, glucose. In all cases, the concentration of glucose in an unknown was determined by comparison with either a 100 mg% or 300 mg% stock glucose standard.

C. Hexokinase Calibration Curve

It was found by titration of the indicator with 0.001 M HCl under the conditions of an experiment that change in the optical density at 560 mμ of 0.033 was equivalent to 1.0 μmol hydrogen ion. The velocities given in the data section are calculated from this result.

LITERATURE REFERENCES

1. Lynen, F., Knappe, J., Lorch, E., Angew., Chem., 71, 65 (1959).

2. Gibson, D.M., Titchener, E.B., and Wakil, S.J., J. Am. Chem. Soc., 80, 2908 (1958).

3. Smith, W.H., J. Path. and Bact., 89, 95 (1965).

4. Williams, V.R., Andrews, D.A., and Christman, J.F., Arch. Biochem. Biophys., 66, 234 (1957).

5. Strauss, R.R., and Moat, A.G., J. Biol. Chem., 233, 765 (1958).

6. Lichstein, H.C., Pro. Soc. Exptl. Biol. Med., 75, 766 (1957).

7. Wagle, S.R., Arch. Biochem. Biophys., 103, 267 (1963).

8. Terroine, T., Arch. Sci. Physiol., 10, 195 (1956).

9. Mistry, S.P., Dakshinamurti, K. Arch. Biochem. Biophys., 96, 674 (1962).

10. Summerson, W.H., Lee, J., Science, 100, 250 (1941).

11. Pilgrim, F.J., Axelrod, A.E., J. Biol. Chem., 145, 237 (1942).

12. Dakshinamurti, K., Arch. Biochem. Biophys., 110, 422 (1965).

13. Gram, M.R., and Okey, R., J. Nutr., 64, 217 (1958).

14. Gibson, D.M., J. Am. Chem. Soc., 80, 2908 (1948).

15. Wakil, S.J., and Titchener, E.B., Biochem. et Biophys. Acta., 29, 225 (1958).

16. Gibson, D.M., and Titchener, E.B., Biochem. et Biophys. Acta., 34, 233 (1958).

17. Wakil, S.J., and Gibson, D.M., Biochem. et Biophys. Acta., 34, 233 (1959).

18. Wakil, S.J., Gibson, D.M., Biochem. et Biophys. Acta., 41, 122 (1960).

19. Wakil, S.J., J. Am. Chem. Soc., 80, 6465 (1958).

20. Brady, R.O., and Gurin, S., J. Biol. Chem., 199, 421 (1964).

21. Kaziro, Y., Leone, E., and Ochoa, S., Proc. Natl. Acad. Sci., 46, 1319 (1960).

22. Halenz, D.R., and Lane, M.D., J. Biol. Chem., 235, 878 (1960).

23. Swick, R.W., and Wood, H.G., Proc. Natl. Acad. Sci., 46, 28 (1960).

24. Guggenheim, K., and Olson, R.E., J. Nutrition, 48, 345 (1952).

25. Broquist, H.P., and Snell, E.E., J. Biol. Chem., 238, 431 (1961).

26. Cheng, A.L., Greenbert, S.M., and Denel, H.J., J. Biol. Chem., 192, 611 (1951).

27. Melnick, D., and Dueul, H.J., Jr., J. Biol. Chem., 239, 211 (1962).

28. Block, K., Science, 150, 19 (1965).

29. Scott, D., Acta Med. Scand., 152, 69 (1958).

30. Barnes, R.H., Kwong, E., and Fala, G., J. Nutrition, 67, 599 (1959).

31. Oxman, M.N., and Ball, E.G., Arch. Biochem. Biophys., 95, 99 (1961).

32. Woesmer, J.F., J. Biol. Chem., 238, 520 (1963).

33. Woesmer, J.F., J. Biol. Chem., 233, 591 (1958).

34. Lynen, F., Fed. Proc., 20, 941 (1961).

35. Sundaram, E.R.B., and Sarma, P.S., J. Sci. Ind. Research, 14c, 793 (1955).

36. Sundaram, E.R.B., Biochem. J., 58, 469 (1954).

37. Dalgliesh, C.E., Biochem. J., 61, 328 (1955).

38. Lindegren, C., and Lindegren, G., Proc. Natl. Acad. Sci., 33, 314 (1947).

39. Chamberlain, N., J. Gen. Microbiol., 7, 54 (1952).

40. Trager, W., J. Biol. Chem., 240, 591 (1963).

41. MacLeod, P.R., and Lardy, H.A., J. Biol. Chem., 179, 733 (1949).

42. Tietz, A., and Ochoa, S., J. Biol. Chem., 234, 1394 (1959).

43. Mistry, S.P., Arch. Biochem. Biophys., 97, 693 (1963).

44. Wood, H.G., J. Biol. Chem., 238, 547 (1963).

45. Kishimo, A., Vitamin, 57, 334 (1958).

APPROVAL SHEET

The thesis submitted by John C. Coggeshall has been read and approved by three members of the faculty of Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

February 21, 1966
Date

Hugh J. MacDonald
Signature of Adviser