STUDIES ON LIVER GLYCOGEN ISOLATED FROM ANIMALS FED DIETS WITH AND WITHOUT ADDED GLYCINE

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INTRODUCTION

An unusual influence of the simple amino acid, glycine, on the carbohydrate reserve of the liver was noted by Todd, Barnes and Cunningham in 1947[1]. In these experiments glycine was incorporated into a control diet by substituting ten to fifteen per cent of the amino acid for an equivalent weight of dextrin. Following the stress of a twenty-four hour fast, it was found that rats fed the glycine experimental ration had liver glycogen levels of around one per cent wet weight and that it exceeded by three times the liver glycogen levels of rats prefed the control diet. This change in behavior of the liver glycogen reserve during stress as elicited by the incorporation of the amino acid into the diet was termed the "glycine effect". Other amino acids that were studied in a similar manner, L-leucine and L-glutamic acid, exerted no such effect while D,L-alanine at a level of 12 per cent in the diet caused a slight increase in the carbohydrate reserve following the stress of a twenty-four hour fast.

The above studies were undertaken to investigate the possible role of individual amino acids in the effect of high protein feeding on glycogen stores as reported by Mirski and coworkers in 1938[2]. These investigators demonstrated that rats which were fed a high carbohydrate diet had a higher liver glycogen reserve than rats fed a high protein diet; but, following a twenty-four hour fast or following a cold

stress of several hours duration, the protein-fed animals had a greater glycogen reserve than the carbohydrate-fed animals. In work experiments, both groups of animals demonstrated a decrease in the level of glycogen; but following recovery periods of four to twenty-four hours and during which no access to food was permitted, the animals fed the high protein diet showed increased levels of glycogen while the carbohydrate-fed animals showed a further decrease in the already depleted glycogen stores. Mirski and coworkers termed this capacity of protein to maintain carbohydrate stores "the protein effect". It was discovered that adrenalectomy abolished this effect of high dietary protein on carbohydrate metabolism. These observations on the effect of high protein diets were confirmed by Neuburger and Brown(3), by Guest(4), and by Todd and coworkers(1).

Todd and Allen studied the effect of dietary glycine on liver glycogen in rats subjected to a cold stress or to a swimming stress(5). It was demonstrated that glycine feeding promoted higher glycogen reserves in the stressed animals than did the control diet. Glycogenesis was more prominent in the glycine-fed animals following a recovery period of one to five hours after the stress.

Further studies were undertaken to elucidate the mechanism of this amino acid in maintaining glycogen reserves

following stress and in stimulating new glycogen formation. Cunningham, Barnes, and Todd[6] studied the effect of glycine feeding and insulin administration on glycogen and blood sugar. No significant difference was found in the levels of liver and muscle glycogen in the two groups of animals after an eight hour fast. At this time twelve units of insulin (Iletin, Lilly) per kilogram body weight were administered subcutaneously. After another five hours the glycine-fed animals showed a decrease in liver glycogen of sixty-one per cent (from 3.51 per cent to 1.36 per cent wet weight). The control animals demonstrated a ninety-six per cent decrease (from 3.89 to 0.13 per cent). Muscle showed similar differences in loss of carbohydrate reserve. The blood sugar level in the glycine-fed animals decreased only twenty per cent while in the control animal it decreased forty-two per cent. The data from these experiments ruled out the possibility that the "glycine effect" results from reduced glycogenolysis, since the post-insulin blood sugar levels in the glycine-fed animals were considerably higher than those of the controls.

Also examined was the possibility that the post-stress extra carbohydrate store of the glycine-fed animals was due to the conversion of this amino acid stored as such or as a constituent of tissue protein. Todd and Talman[7] reported on studies of analyses of free and total glycine in blood,

liver, muscle, intestine, and kidney. The data indicated that even if all the extra glycine in the body of glycine-fed rats were converted completely to carbohydrate, it would not be sufficient to account for the glycogen difference in the glycine-fed and control-fed rats following a fast or following an insulin stress.

Earlier work on the conversion of glycine to liver glycogen included that of Lusk(8) who found a quantitative conversion of ingested glycine into urinary glucose in phlorhizinized dogs. In 1933 MacKay and coworkers reported that the peak of glycogen formation occurs fourteen hours after glycine ingestion, thus suggesting an indirect mechanism.

Olsen, Hemingway, and Nier(9) administered to fasted mice glycine labelled with the stable isotope of carbon in the carboxyl group. After sixteen hours, it was found that the liver glycogen level was elevated but the total increase in glycogen was not due to conversion of glycine to carbohydrate since only one per cent of the fed isotope was present in the isolated glycogen.

Lockett and Evans(10) reported that glycine injected subcutaneously caused a reduction in hepatic glycogen in intact mice and in adrenalectomized cortisone-treated mice. In salt-maintained and in desoxycorticosterone treated

adrenalectomized mice glycine exerted a glycogen sparing action equivalent carbon for carbon to that of glucose. A possible explanation for these observations is that glycine administered subcutaneously to a mouse could act as stress leading to glycogenolysis and that the sparing action was masked in the first group of animals.

Hess and Shaffron[11] studied the rate of absorption and the formation of hepatic glycogen when glycine was stomach-tubed to fasting rats. Their studies showed that glycine was definitely gluconeogenic, reaching a peak at fourteen hours following the administration of glycine. A smaller peak was observed at one hour and probably represents the ability of glycine to form glycogen directly. The peak observed at fourteen hours was not attributable to delayed absorption of glycine from the gastro-intestinal tract.

Observations made by Dakin[12] caused him to remark that glycine disturbs the equilibrium that exists among amino acids or peptides in the body and results in the liberation of amino acids that are capable of furnishing glucose units.

The fact that adrenalectomy abolishes the "glycine effect" in the rat combined with the observations on adrenal cortex-carbohydrate-protein interrelationships led Talman[13] to study the urinary excretions that reflect end products of

protein metabolism in the rat. The experiment was designed so that it was possible to determine the amount of nitrogen ingested and excreted with a high degree of accuracy. Animals that received the glycine diet retained significantly more nitrogen than the control-fed animals during the first day of feeding. But when the rats were stressed with a twenty-four fast the animals prefed the glycine diet showed a significantly greater net negative nitrogen balance. This increased nitrogen excretion was in excess of the amount of glycine-nitrogen received in the diet and all other dietary nitrogen. The results were interpreted as indicating that glycine feeding resulted in increased adrenal cortical activity.

However, unpublished data on the classical parameters of adrenal cortical activity -- ascorbic acid and cholesterol levels of adrenal gland after increased activity -- fail to show any significant difference between the experimentally-fed and control-fed animals after a fasting stress. It is apparent that ascorbic acid and cholesterol levels do not show a significant decrease in response to two days of glycine feeding and the mild stress of fasting. Study of urinary formaldehydogenic steroids did not aid in the solution of the possible mechanism of the "glycine effect".

The accumulation of glycogen depends upon many reactions,

any of which may be limiting and an explanation for the physiological phenomenon may require evaluation of all of these factors[14]. The nature of the glycogen molecule is a factor to be considered in searching for an explanation of the "glycine effect".

When glycogen is hydrolyzed completely, it yields alpha-D-glucose. Only two linkages are found in glycogen -- the alpha-1,4- and the alpha-1,6- glucoside linkages. This was arrived at by a detailed study of dimethyl, trimethyl, and tetramethyl glucose units derived from purified methylated glycogen by Haworth and associates[15]. In 1954 additional confirmation on the presence of only these two linkages was presented by Bell and Manners[16]. The linear portion of the branched chains of the polysaccharides are made up of 1,4 linked glucose residues. Branching occurs through a 1,6-linkage as illustrated in Figure 1.

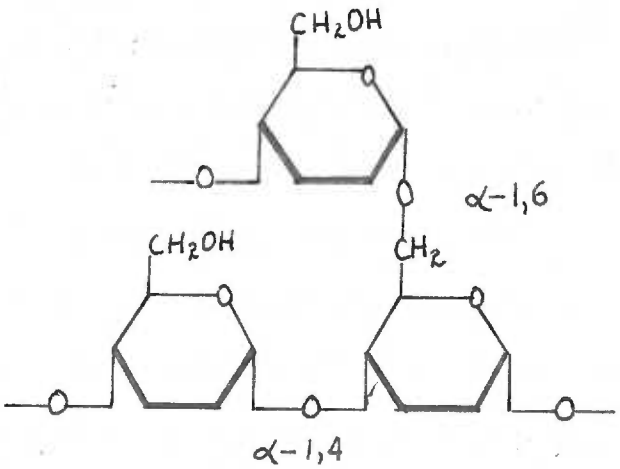
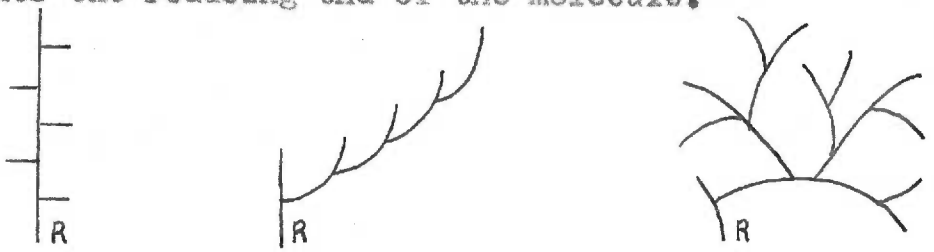


Figure 1. Illustrating the alpha-1,4- and alpha-1,6-linkage in the glycogen molecule.

Three models had been proposed for the structure of glycogen. Staudinger and Husemann[17] proposed a comb-like structure in which each side branch arises from a main chain. On the basis of methylation studies on glycogen and on end group analyses, Haworth[18] offered the proposal that glycogen is a laminated structure in which each branch arises from a preceding branch. Meyer and coworkers[19] examined the properties of the limit dextrin obtained by the action of beta-amylase. Acetates of these molecules, if glycogen has a laminated structure, should yield elastic films on evaporation of a solution on a glass plate. But the beta-amylase limit dextrin of glycogen exaporated on a glass plate could not be detected as a film. These observations favored the irregular, tree-like structure as proposed by Meyers' group. Staudinger, commenting on the work of K. H. Meyer stated that his work had anticipated the arrangement of glucose residues in glycogen as a tree-like structure.[20]. Figure 2 shows the three models proposed for the structure of glycogen. R represents the reducing end of the molecule.



Staudinger[17] Haworth[18] Meyer[19]

Figure 2. Various models proposed for the structure of glycogen.

Cori and coworkers[21] finally established the tree-like structure by enzymatic dissection of the glycogen molecule. There is only one socalled "reducing end" in the molecule. All the remaining terminal glucose units are nonreducing. It can be demonstrated that the number of terminal groups is one greater than the number of branch points. There are available three independent methods for determining the degree of branching (that is, the number of alpha-1,6 linkages in relation to the number of alpha-1,4 linkages). These methods are 1) the methylation technique based on the work of Haworth, 2) the periodate oxidation of glycogen as described by Potter and Hassid[22], and 3) the enzymatic method developed by Cori and Larner[23]. The values obtained by the three methods for the average number of glucose residues in the outer branches are in general agreement[21]. In the chemical methods the percent of end groups can be determined because in the methylation technique the nonreducing terminal groups yield 2,3,4,6-tetramethylglucose and with periodic oxidation each terminal group yields a molecule of formic acid. The number of end groups is related to the number of branch points as n is to n - 1. Only the enzymatic method can determine the number of glucose residues in the alpha-1,6 linkage; these molecules are split off by amylo-1,6-glucosidase specifically as free glucose. The other glucose molecules are split off

as glucose-1-phosphate through the action of phosphorylase.

As knowledge of the glycogen structure increased, a better understanding of the processes of synthesis and degradation of glycogen developed. The three enzymatic reactions involved in the conversion of glucose to glycogen include:

(1) glucose + ATP* = glucose-6-phosphate + ADP*

(2) glucose-6-phosphate = glucose-1-phosphate

(3) glucose-1-phosphate = glycogen + inorganic phosphate

Sum: glucose + ATP → glycogen + inorganic phosphate.

The first reaction is called the hexokinase reaction; since there is a large decrease in free energy in this reaction, the equilibrium favors the formation of glucose-6-phosphate. A different enzyme is required to convert glucose-6-phosphate to glucose in the formation of blood sugar from glycogen reserves in the liver[24]. Reactions 2 and 3 are readily reversible since there is little change in free energy. Reaction 2 is known as the phosphoglucomutase reaction in which the phosphate group is shifted from carbon six to carbon one of the glucose with the glucose-1,6-diphosphate serving as the intermediate. This reaction was described by Leloir[25]. Phosphorylase adds glucose-1-phosphate units in a random manner to the outer branches of the glycogen molecule; this is illustrated by reaction 3. The linkage formed is of the alpha-1,4 variety;

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* ATP = adenosine triphosphate; ADP = adenosine diphosphate.

inorganic phosphate is liberated, and the polysaccharide chain grows in length. Polysaccharides must be present in an in vitro reaction if phosphorylase and glucose-1-phosphate are to synthesize glycogen. When a new branch point is to be established, amylo- (1,4 - 1,6)-transglucosidase is required(26). This may be represented schematically as follows:

where O represents a glucose unit.

In the course of glycogen degradation, phosphorylase in the presence of inorganic phosphate lyses the alpha-1,4 linkage and liberates the hexose moiety as glucose-1-phosphate. As a branch point is approached, the glucose residues involved in the alpha-1,6 linkage are exposed and offer an obstruction to the further action of phosphorylase. In order to remove this obstacle to the process of phosphorolysis, this branch point must be hydrolyzed by a debranching enzyme, amylo-(1,6)-glucosidase(27), thus liberating free glucose.

With two enzymes as dissecting tools, the group in the Coris' laboratory was able to carry out stepwise degradation of glycogen by the action of muscle phosphorylase and amylo-(1,6)-glucosidase(21). The limit dextrins formed by the

action of phosphorylase were isolated and then incubated with glucosidase. Determination of glucose-1-phosphate was carried out after the action of phosphorylase and indicated the average length of the outer chain. The free glucose determined after glucosidase had reacted gave information regarding the number of branch points. After the third phosphorylase step the liver glycogen was degraded seventy-seven per cent. After the fourth phosphorylase-glucosidase cycle the glycogen was degraded eighty-eight per cent. The per cent of total branch points present in each tier became progressively less as the reducing end was approached. Only one type of the models proposed for glycogen fit the analytical data, that of a multi-branched tree-like structure as originally proposed by Meyer. A portion of the glycogen molecule as it is stepwise degraded is diagramed in Figure 3.

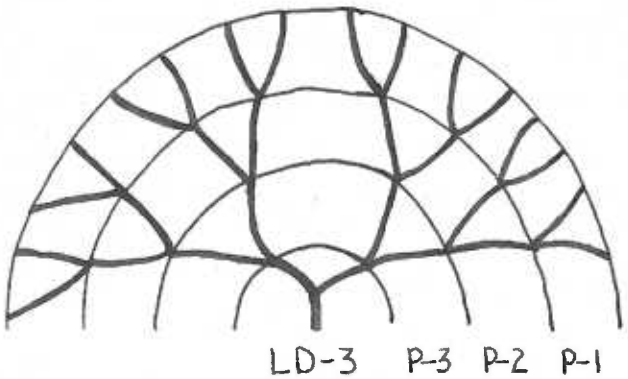

Figure 3. Diagram shows the tiers removed by three phosphorylase cycles (P-1, P-2, P-3) and the resulting limit dextrin (LD-3).

Von Gierke's disease or glycogen storage disease is characterized by an enlargement of liver and kidneys with extensive deposition of glycogen in these organs. Leading to the eventual downhill course of children with this syndrome is the unavailability of their reserves of glycogen for 1) maintaining the blood sugar level, 2) making available sources of energy for synthetic processes in protein and fat metabolism, and 3) providing a store of reserve energy which may be made available in times of stress[28]. Schoenheimer demonstrated that glycogen isolated from von Gierke's original case could be degraded by minced normal human liver -- thus suggesting an enzyme deficiency for normal breakdown in the diseased state[29]. Using endgroup analyses on glycogen samples isolated from liver of patients with glycogen storage disease, Cori was able to demonstrate that eight of ten cases possessed glycogen of normal structure, but lacked entirely or were deficient in glucose-6-phosphatase[30]. The remaining two cases showed definitely abnormal structure; the glycogen molecule in one case resembled a limit dextrin while the other case had a liver glycogen that was less branched and possessed longer inner and outer chains than normal glycogen. Recant has presented an excellent review of glycogen storage disease[31]. It is now recognized that there are at least four glycogen storage diseases.

The size and shape of the glycogen molecule in tissue is dependent upon the ratio of three enzymes, namely, phosphorylase, the branching enzyme, and the debranching enzyme. The types of glycogen storage disease reflect a variation in the activity of these enzymes plus that of glucose-6-phosphatase. Thus, many of the laboratory findings may be explained on the pattern of enzyme activity(32). In the classical von Gierke's disease where the only organs involved are the liver and kidney, the structure of glycogen is normal. It is the lack of or the deficiency of glucose-6-phosphatase that causes the levels of glycogen reserve to remain at high levels and be unavailable for tissue metabolism. Hence, findings in the peripheral blood will include a marked hypoglycemia, possibly a ketosis and a hyperlipemia. When epinephrine is employed to test the mobilization of glycogen, the response as measured by an increase in blood glucose is negative or slight, depending on the activity of the phosphatase.

In glycogen storage disease of the heart, the structure of glycogen appears to be normal. A generalized glycogenosis is found, but most stirking is the infiltration of cardiac muscle and of skeletal muscle, especially of the tongue and the diaphram. Clinically, patients may resemble mongolism of cretinism(33). These patients do not demonstrate a

hypoglycemia, ketosis, or hyperlipemia, and their response to epinephrine is normal. One may draw the conclusion that glycogen is available for use by the body and that there exists no abnormal pattern enzymatically for synthesis and degradation of glycogen. But why does glycogen accumulate?

Another type of glycogen storage disease involving the liver and the reticulo-endothelial system may have liver polysaccharides that resemble amylopectin, that is to say, a molecule with fewer branch points and longer end chains than glycogen. These findings are suggestive of a deficiency of the branching enzyme, not of glucose-6-phosphatase. Therefore, no changes in the peripheral blood such as hypoglycemia are noted. The epinephrine response may be moderate and delayed. The glucose tolerance curve shows a rise and then a slow fall in the concentration of blood sugar(34).

Forbes(35) and Hartmann(31) have reported cases of glycogen storage in liver and muscle. Isolated glycogen examined by Cori(30) was found to be abnormal in structure since it possessed numerous short outer branches and resembled a limit dextrin. Phosphorylase activity apparently was normal; a deficiency in the branching enzyme existed. Therefore, the outer tier of the molecule was accessible to phosphorylase and glucose units could be easily added and

removed. The inner core was unavailable. Patients exhibited a mild hypoglycemia and acetonemia; they showed a definite but small response to epinephrine.

Illingworth, Larner and Cori[36] examined the structure of normal rabbit glycogen in various states of nutrition. Fasted rabbits were infused with glucose or fructose solutions. The outer branches of rabbit glycogen are short when the glycogen content is low; these branches increase in length as new glycogen is deposited. These differences in chain length may be explained on the basis that the ratio of phosphorylase activity to branching enzyme favors the formation of straight chains. As the length of time increases following the period of feeding, the ratio favors the action of branching enzyme and a highly branched glycogen molecule develops, but the branches are shorter in length. It can be said that there is alteration in the structure of the glycogen both in physiologic and in pathologic states; the pattern of the alteration can help to predict the findings in the peripheral blood and the changes that occur during stress.

These changes in branch length and in degree of branching may be responsible for changes in physical properties of glycogen such as solubility. Early workers in the field of carbohydrate metabolism realized that tissue glycogen could not be extracted completely by hot or cold water and by acids

such as acetic, hydrochloric and trichloroacetic. In 1892 it was reported by Frankel that an extraction method using trichloroacetic acid did not dissolve the total amount of tissue glycogen(37). This was found to be true also for water extractions by Loeschcke(38). In 1906 Pflüger described a method involving alkali digestion to remove the glycogen quantitatively from tissues. Its chief disadvantage is the difficulty in isolating a glycogen product that is free from the alkali and the protein degradation products. The modification of this method by Somogyi is used today in the laboratories of Cori(36), Larner(39), Smith(40) and others for the isolation of glycogen. A point of controversy still exists in the literature regarding the stability of the glycogen molecule in alkali. Bell and Young(41) reported that purified preparations of glycogen obtained by hot water extraction or by the Pflüger hot alkali extraction method did not exhibit any differences in the properties measured such as specific rotation, reducing power, coloration with iodine and behavior in the polarimeter during the process of hydrolysis.

In 1933 Chiao Tsai(42) demonstrated that with repeated aqueous extractions of rabbit's liver there always remained a small quantity of glycogen in the tissue residue. Carruther and Ling(43) confirmed these observations, but they

also stressed the quantitative aspect in that the hot water-extractable fraction of glycogen was relatively constant irrespective of the variation in the total glycogen.

It was Bloom who directed attention toward the quantitation of glycogen fractions in tissues. In a study of phosphate fractions in liver and muscle, it was noted that a significant concentration of glycogen was readily extractable by cold trichloroacetic acid, and that this fraction of the total glycogen was quite constant(44). This observation was extended to a critical survey of the behavior of glycogen in rat liver and muscle under various physiologic situations(45). The trichloroacetic acid-extractable glycogen comprised eighty-five per cent of the total glycogen of the liver and fifty-five per cent of the total muscle glycogen in the fed rat. During a fast, the acid-extractable fraction disappeared more rapidly than total glycogen. After a twenty-four hour fast it was almost completely depleted. Upon the feeding of glucose to fasted rats, the acid-extractable fraction was quickly reconstituted. Hence, it occasionally is called the labile fraction of glycogen. The difference between the total glycogen of a tissue and the trichloroacetic acid-soluble fraction represents the nonacid-extractable glycogen or residual glycogen to which earlier reference has been made(42, 43). This fraction of glycogen was not altered

greatly by changing physiologic states in the rat.

Previous work in this laboratory(46) was carried out to determine if glycine feeding could alter the levels of liver and muscle fractions of glycogen. It was found that the action of dietary glycine was to increase the acid-extractable glycogen. This elevation accounted for the observations that liver glycogen levels were three times that seen in the control-fed animals following a twenty-four hour fast. The increase in liver glycogen following the administration of adrenal cortical extract to intact rats during a period of fast was accounted for mainly by an increase in the acid-extractable glycogen.

It is possible that glycine feeding plus the stress of fast may evoke a mechanism in polysaccharide formation in the rat that results in an altered glycogen molecule with different physical and chemical properties compared with glycogen in the control animals. This alteration in the glycogen molecule, if found, might explain the maintenance of high carbohydrate reserves in the liver during stress and its availability for blood sugar formation.

It is proposed to present studies designed to investigate alterations of the glycogen molecule as revealed by 1) observations on the sedimentation rate of glycogen in the analytical ultracentrifuge and 2) dissection with beta-

amylase to indicate the degree of branching in the outer tier of the glycogen molecule and the size of the resulting limit dextrin. These measurements were chosen in hopes of adding information to help explain the mechanism of the "glycine effect".

Glycogen was isolated from pooled livers of animals fed diets with and without glycine. These animals had been subjected to a fast of zero, sixteen or twenty-four hours. The isolation techniques for tissue glycogen included both the hot alkali method and the cold trichloroacetic acid procedure. These methods of isolation were chosen in an attempt to compare the effects on glycogen structure as a result of experimental glycine feeding and to examine the question of destruction of glycogen by hot alkali.

Lastly, it is known that glycogen can be separated into several fractions by electrodialysis. Using bridge electrophoresis, differences in the behavior of various isolated glycogens were studied. The possible separation of the glycogen samples into two components that would correspond to the labile and the residual fractions was evaluated.

EXPERIMENTAL

Animals: Male Sprague-Dawley of 200 to 300 grams were used for the preparations of liver glycogen samples. The weights within the experimental groups were matched.

Rations: Rats were maintained on Purina Laboratory chow for several days prior to the feeding of the synthetic rations. This was to insure that these animals were in a good state of nutrition prior to the beginning of the experiment. The compositions of the control and glycine rations are shown below:

	Control Ration Percent	Glycine Ration Percent
Casein	16	16
Yeast (Squibb)*	10	10
Salt mixture(47)	5	5
Cod liver oil	2	2
Wesson oil	5	5
Dextrose	8	8
Dextrin	54	44
Glycine	0	10

It will be noted that the composition of the control and

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* 4.3 per cent protein was supplied by 10 per cent Squibb's Brewer's Yeast.

glycine diets are identical except that glycine was substituted for an equal weight of dextrin in the control diet. The total amount of protein in the diet was twenty per cent, supplied by the casein and by the yeast. These rations supplied the rat with sufficient protein of good quality. Vitamins were supplied by the yeast and the cod liver oil.

The daily food intake of the control- and glycine-fed animals was between fifteen and twenty grams per twenty-four hours.

Feeding routine: The rats were placed in individual cages which were equipped with screen floors to prevent coprophagy. The synthetic rations were offered in large food cups equipped with a wide base in order to minimize spillage and contamination of food. The animals received twelve to fifteen grams of the control ration for each of two days in order to accustom them to a powdered synthetic diet. This procedure also allowed better control of the food intake during the next two days.

In the late afternoon when beginning the two day experimental feeding period, the animal was offered twenty grams of the glycine ration or continued on this amount of the control ration. On the following afternoon the animal was offered fifteen grams of the ration being consumed. Five hours before the end of the forty-eight hour feeding

period the food can was removed; three hours later, five grams of ration was offered to the rat. This procedure was employed to regulate the length of fasting. The final two hour feeding period occurred at a time of day when the rat resumed activity and feeding. After completion of the final feeding, the rats were fasted for periods of zero, sixteen or twenty-four hours. Then they were removed from the cage, weighed, and sacrificed for the isolation of liver glycogen.

Isolation of glycogen: The animals were anesthesized with Nembutal (5 mg. per 100 grams body weight). When surgical anesthesia was obtained, an abdominal incision was made in the midline, the liver was grasped and rapidly freed from the surrounding tissue. It was then blotted to remove as much blood as possible. The liver was placed on a square of Parafilm and quickly minced with a pair of sharp scissors and placed in sodium hydroxide or in cold trichloroacetic acid solution. Pooled liver samples were used in order to obtain a generous supply of purified glycogen. Random liver samples were used to check the liver glycogen levels by the method of Good, Kramer and Somogyi(48). The tissue sample was digested in 30 per cent potassium hydroxide in a boiling water bath; then 95 per cent alcohol was added to yield a final concentration of 55 per cent to precipitate the glycogen. This precipitate was washed with ethanol. The glycogen was

hydrolyzed for two and a half hours by N sulfuric acid on a boiling water bath. The Shaffer-Hartmann method was employed to determine resulting glucose. Glycogen concentrations were expressed as grams of glucose per one hundred grams of liver wet weight.

The hot alkali method employed for glycogen isolation was essentially that described by Somogyi for the preparation of a phosphorus- and nitrogen-free glycogen(49). The pooled liver sample was introduced into a 250 ml. Erlenmeyer flask containing 40 per cent sodium hydroxide and fitted with a rubber stopper through which a length of capillary tubing extended and served as an air reflux. This flask was then immersed in a boiling water bath for three hours: during the first hour of heating the flask was agitated. Following the digestion period, the flask was removed from the bath and allowed to cool at room temperature without disturbance. On cooling, a solid layer of soap formed on the surface from which the gelatinous fluid below was decanted off. The removal of soaps facilitated the purification procedures. A small amount of glycogen was discarded with this soap. The gelatinous fluid was measured and to it was added sufficient 95 per cent ethanol to yield a final alcohol concentration of 33 per cent. The precipitate was allowed to settle overnight at room temperature and was then separated by centrifugation for fifteen minutes at 1500 r.p.m.

This precipitate was washed in the centrifuge tube with a solution consisting of one volume of 95 per cent alcohol to two volumes of 20 per cent sodium hydroxide until the washing fluid was nearly colorless. The moist impure glycogen was dissolved in water, and the insoluble particles removed by filtration. The filtrate was placed in a 40 ml. centrifuge tube and kept in an ice water bath while 2 N hydrochloric acid was added dropwise until it gave an acid reaction with Congo paper (pH 4 to 5). This last step was carried out only when it became difficult to free the glycogen from the last traces of contamination. The precipitate that formed was removed by filtration. Immediately an equal volume of ethanol was added, the precipitate was allowed to form and was collected by centrifugation. This glycogen was progressively dehydrated with successive washings, two times each with 55, 70, and 95 per cent ethanol, once with absolute alcohol, and finally with anhydrous diethyl ether. After the last washing, the centrifuge tube was placed at a slant, mouth down, in order to enhance the evaporation of ether. The glycogen was obtained as a fine powder.

The second means of isolation of glycogen involved the grinding of liver with cold 10 per cent trichloroacetic acid solution and the subsequent extraction of the residue with 5 percent trichloroacetic acid solution as described by Stetten

and Stetten(50). The grinding was carried out in the cold room (- 2° C.) with a chilled mortar and pestle and sand. The residue and sand were removed by filtration. The glycogen was precipitated immediately with an equal volume of 95 per cent ethanol and collected by centrifugation. It was necessary to allow acid-alcohol solutions of glycogen obtained from the livers of control rats fasted twenty-four hours to remain overnight in the cold room in order to allow the precipitate to form. The glycogen obtained by centrifugation was dissolved in distilled water and reprecipitated several times with an equal volume of ethanol. The glycogen then was subjected to the dehydration and washing procedure described above. Occasionally, the addition of lithium bromide was required to increase the electrolyte concentration and induce flocculation.

The Kjeldahl method was used to determine nitrogen. One hundred milligrams of glycogen was dissolved in 12 ml. of distilled water. In order to determine the concentration of glycogen, 1.0 ml. was hydrolyzed in N sulfuric acid in a boiling water bath. To 10.0 ml. of the glycogen solution one ml. of selenium digestion mixture was added. When the digestion was about complete, a few drops of 30 per cent hydrogen peroxide was added, and the heating continued for an additional ten minutes. The determination of nitrogen was

carried out by distillation of the ammonia into 1/70 N sulfuric acid and back titration with 1/70 N sodium hydroxide, using Tashiro's indicator. Each ml. of the 1/70 N sulfuric acid is equivalent to 0.2 mg. of nitrogen. Due to limited quantities of glycogen isolated, it was not possible to check the nitrogen content of all the samples isolated. The glycogen present was determined as glucose by the Shaffer-Hartmann method.

Methods for examination of the glycogen molecule:

1. Beta-amylase degradation.

A time degradation curve of the action of beta-amylase on glycogen was obtained as follows: one hundred milligrams of glycogen was dissolved in 28 ml. of distilled water containing one drop of 40 per cent sodium hydroxide. In order to determine the concentration of the glycogen solution, 2 ml. was hydrolyzed by N sulfuric acid, and the glucose determined by the Shaffer-Hartmann procedure. One hundred milligrams of beta-amylase was dissolved in an acetate buffer of p^H 4.8. The acetate buffer was prepared by dissolving 30.7 grams of sodium acetate trihydrate in distilled water; 10.2 ml. of glacial acetic acid was added and the p^H was adjusted to 4.8 with the aid of a p^H meter. The final volume of the buffer was 250 ml.(51). In order

to obtain optimal and rapid degradation without complications due to the formation of associated enzyme-resistant particles, 25.0 ml. of the dilute alkaline solution of glycogen was added slowly with stirring to the concentrated solution of beta-amylase in a 150 ml. Erlenmeyer flask. Incubation of this reaction mixture at 37° C. was continued for ten hours.

Aliquots of 1 ml. were taken at intervals for analysis of the amount of maltose freed by the action of enzyme. 1 ml. of distilled water was added to the aliquot followed by the addition of 2.0 ml. nitrosalicylic acid reagent. The color reagent was prepared by dissolving 1 gram of 3,5-dinitrosalicylic acid (Eastman Organic Chemicals) in a small quantity of warm distilled water, adding 20 ml. of 2 N sodium hydroxide solution and 30 grams of Rochelle salts, and diluting to a volume of 100 ml. This solution was prepared on the day of use to eliminate alterations in sensitivity caused by the absorption of carbon dioxide(52).

The color reagent and maltose-containing solution were heated for five minutes in a boiling water bath. After cooling, the solution containing the brown reduction product was diluted to 20 ml. with distilled water. The color intensity was measured at 540 λ in a Coleman Junior Spectrophotometer. Calculations were made from a standard curve developed with pure maltose. The control blanks of glycogen

alone or enzyme alone showed no color reaction with the nitrosalicylic acid reagent.

The degradation of glycogen by beta-amylase was almost complete at the end of three hours; the results agree with those of Stetten and Stetten(53). The experiment indicates that the rate of beta-amylase gradually slows as it approaches the alpha-1,6 linkage. A graph of the time curve is shown below:

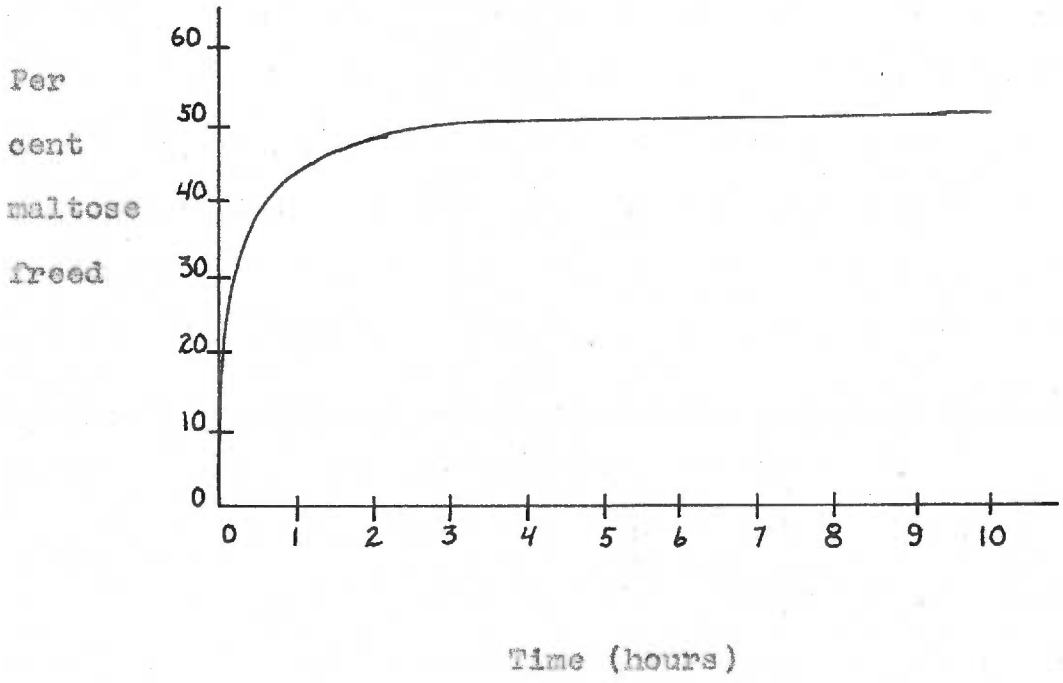


Figure 4. A time course study of the degradation of glycogen by beta-amylase to free maltose.

On the basis of the above findings, it was decided to stop arbitrarily the action of beta-amylase on the various

glycogen samples after five hours incubation. The reaction mixture was transferred to a volumetric flask and diluted to 100 ml. with distilled water. 2 ml. of this solution was added to 2 ml. of nitrosalicylic acid reagent in order to determine the concentration of maltose as previously described.

Limit dextrin was prepared as follows: the enzyme and glycogen were allowed to react for 24 hours at 37° C. Then the solution was made alkaline with sodium hydroxide, neutralized, and more enzyme added. The incubation was repeated. The limit dextrin was precipitated by the addition of two volumes of methanol and washed with 65 per cent methanol.

This limit dextrin was subjected to the action of beta-amylase as described for glycogen except that the incubation time was extended to twenty-four hours. The absence of reducing sugar indicated the absence of alpha-amylase in the beta-amylase preparation used(54). Contamination of the enzyme with alpha-amylase would yield results impossible to interpret due to the combined action of the two enzymes.

2. Ultracentrifuge studies.

The sedimentation behavior of glycogen was studied in the Spinco model "E" ultracentrifuge. This instrument

furnishes an accurately controlled centrifugal field to which the solution under observation is subjected. Furthermore, this solution is located in an optical cell of a rotor which can be operated free from vibration and from fluctuations in temperature because the drive unit, the rotor chamber, the optical system, and the camera form a structural unit that is shock mounted.

All the runs were conducted at a speed of 20,400 r.p.m. at room temperature. Observations were made on a one per cent glycogen - one molar sodium chloride solution in a Type An-A rotor. The refractive index system of the Philpot-Svensson type is utilized in the Spinco ultracentrifuge.

A typical experiment included the assembly of the optical cell and the filling of the cell with the freshly prepared solution to be examined. The rotor was prepared by the alignment of the optical cell and its counterbalance. It was then attached to the drive and the rotor chamber evacuated to a pressure of less than one μ. The rotor was accelerated to the desired operating speed. When equilibrium was obtained, the movement of the sedimenting boundary was recorded by the camera automatically at intervals of 480 seconds. At the same time observations were made on the temperature of the reference thermometer and of the rotor chamber, the operating speed, and the

inclined angle of the schlieren diaphragm. At the completion of the run, the behavior of the sedimenting boundary had been recorded sequentially on the photographic plate emulsion which was then developed. Measurement was made of the distance the peak of the sedimenting boundary had moved from the center of rotation in a given time interval divided by the average centrifugal field over this interval(55). This is represented mathematically as:

$$s = \frac{(x_2 - x_1)}{(t_2 - t_1)\, w^2\, x_m}$$

where $(x_2 - x_1)$ = the distance travelled by the sedimenting boundary during the interval.

w^2 = the average angular velocity of the rotor system in radians per second or $(\text{r.p.m.} \times \frac{2\pi}{60})$

$x_m = \frac{(x_2 + x_1)}{2}$ = average position of the sedimenting boundary during the interval

In order to compare the sedimenting behavior of solute molecules, the sedimentation constants were corrected to a constant state, i.e., to the conditions which would be obtained in a hypothetical medium of water at 20° C. This correction formula may be expressed as:

$$s_{20,w} = s_{obs.} \left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta}{\eta_o}\right)\left(\frac{1-\bar{V}\rho_{20,w}}{1-\bar{V}\rho_t}\right)$$

where $\left(\frac{\eta_t}{\eta_{20}}\right)$ = the principle correction factor corresponding to the viscosity of water at t° relative to that at 20° C.

$\left(\frac{\eta}{\eta_o}\right)$ = the relative viscosity of 1 M NaCl solution to that of water.

$\rho_{20,w}$ = density of water at 20° C.

ρ_t = density of 1 M NaCl solution at t°

$\bar{V}$ = the partial specific volume of glycogen. The value used for these calculations was 0.65 as determined by Bridgman(57).

The constants ($s_{20,w}$) are expressed in Svedverg units where $S = 1 \times 10^{-13}$.

3. Electrophoresis studies:

The power for electrophoresis was supplied by a high voltage regulator Model 1910 manufactured by Research Specialities Company. It was capable of delivering up to 100 milliamperes direct current between 50 and 600 volts. Platinum electrodes were employed. The bridge was prepared as described by Briggs, Garner, Montgomery and Smith(58). It

was composed of two glass plates (10 cm. x 29 cm. x 0.6 cm.) sandwiching two plastic sheets of like dimension. The glass fiber filter paper(59) was supported between the plastic plates. Rectangular plastic dishes contained the electrolyte reservoir and the electrodes.

The procedure employed was as follows: 0.1 milligram of glycogen in 1λ was spotted on the point of origin, which was located two-thirds of the distance from the compartment that contained the anode. It was dried with the aid of warm air. The paper was then wetted by dipping one end in 2 N sodium hydroxide solution to within one-half inch of the point of origin. It was then blotted to remove the excess of sodium hydroxide solution. A similar maneuver was carried out for wetting the other end of the paper. The glass fiber paper was inserted between the two plastic sheets and the assembly of the bridge completed. Pressure was applied by clamps. The ends of the paper were allowed to dip into the sodium hydroxide reservoir. Ten minutes was allowed for equilibrium after the two liquid fronts met at the point of origin. A potential of 100 volts was then applied for a period of twelve hours at an operating current of 70 milliamps. At the completion of the electrophoresis process, the paper was removed and dried in air. Glycogen was located by spraying the glass fiber with a solution

composed of 0.5 per cent potassium permanganate in N sodium hydroxide(60). At the completion of the reaction between glycogen and permanganate ion in an alkaline solution, it was possible to wash away the excess staining solution. There remained a brown stained area which indicated the distance the glycogen had traveled from the point of origin under the influence of an electrical field. This distance was measured in centimeters.

RESULTS AND DISCUSSION

The Kjehdahl method revealed that the glycogen samples isolated by hot alkali or trichloroacetic acid methods contained less than 0.05 per cent nitrogen. This indicated a high degree of purity of the glycogens used in the following studies.

Glycogen degradation by beta-amylase: Table 1 presents a tabulation of the percent degradation of glycogen by beta-amylase. The glycogen was isolated by hot alkali or by cold trichloroacetic acid from pooled liver tissue of rats fed the control or the glycine ration. The rats had been subjected to a fast of 0, 16, or 24 hours.

The action of beta-amylase is tosplit only the second alpha-1,4-glucosidic linkage from a nonreducing chain end of the glycogen molecule, thus liberating a maltose residue. Beta-amylolysis eventually stops when an alpha-1,6-glucosidic linkage is encountered. Sumner and French have offered evidence that beta-amylase attacks the outer chain lengths of glycogen until its action is stopped within 1.5 glucose units of the branch point(61).

The degradation by beta-amylase of hepatic glycogen prepared from unfasted rats fed the glycine or the control diet ranged from 54 to 58 percent. The average enzymatic degradation of glycogen obtained from rats fasted 16 hours ranged from 53 to 56 percent. With glycogen samples

Table 1.

The degradation by beta-amylase of hepatic glycogen isolated from rats fed the glycine or the control diet and subjected to various lengths of fast.

	Glycogen extracted by hot alkali		Glycogen extracted by cold trichloroacetic acid	
Fast (hours)	Glycine-fed (% degradation)	Control-fed (% degradation)	Glycine-fed (% degradation)	Control-fed (% degradation)
0	54 ± 6* (3)**	58 ± 4 (6)	54 ± 2 (4)	57 ± 3 (4)
16	53 ± 2 (4)	56 ± 1 (4)	54 ± 4 (4)	54 ± 1 (4)
24	55 ± 3 (4)	56 ± 1 (4)	53 ± 3 (4)	50 ± 7 (3)

*Standard deviation.

**Number of pooled liver glycogen samples studied included in parentheses.

obtained from rats subjected to a 24 hour fast, the average degradation by beta-amylase was 50 to 56 percent. No significant difference in degradation was found in glycogens isolated by the two procedures. From these results, the following conclusions can be drawn: 1) glycine feeding did not cause a difference in the degree of branching of glycogen; 2) fasting did not affect the chain lengths of glycogen; and 3) the methods used for the extraction of tissue glycogen did not affect appreciably the degree of branching as determined by beta-amylase.

From recent studies in the synthesis and breakdown of glycogen, the lack of difference of glycogen isolated from animals in different fasting states is not unexpected. It has been postulated that the branching enzyme transfers part of a glycogen chain after it has been lengthened by the action of phosphorylase and has reached a critical length, thus forming a new branch point. Long chains are permitted to exist briefly. Debranching enzyme may be envisioned to become more active when phosphorylase is removing outer chains of glycogen as such during periods of fasting. Amylo-1,6-glucosidase removes the branch point and allows branches to become available for the action of phosphorylase. Therefore, the degree of branching and the chain length of a glycogen molecule is the product of the relative rates of

activity exhibited by phosphorylase, branching enzyme, and debranching enzyme, and these are influenced by the nutritional and hormonal status of the animal. It would seem that size of branches available to the action of beta-amylase would be similar in glycogen of fed and of fasted rats. Illingworth, Larner and Cori were able to demonstrate that the degree of branching of liver glycogen obtained from a rabbit that had been infused with a glucose or fructose solution was greater than that determined for glycogen obtained from a fasted rabbit. The infusion of hexose probably caused the action of phosphorylase to predominate while the branching enzyme was not sufficiently active to shorten the lengthened branches at the time the liver was removed for the isolation of glycogen.

In 1952 Bell and Manners[62] reported that hydrolysis by beta-amylase normally resulted in 45 ± 5 percent conversion to maltose when the glycogen had a chain length of approximately twelve glucose residues. In this study glycogen was obtained from beef and rabbit liver, mussels, and corn. A sample of rabbit liver glycogen was degraded 53 per cent by beta-amylase; its chain length was 18 glucose units. The glycogen had been isolated by the Pflüger method or by hot aqueous extraction and purified by precipitation with glacial acetic acid. Stetten and Stetten[50] have

questioned the use of acid in the isolation of glycogen and have shown that brief contact of cold trichloroacetic acid in their preparations resulted in some degradation of glycogen. The use of acetic acid in the purification procedures may cause degradation of glycogen and result in decreased degradation by beta-amylase.

In 1954 Stetten and Stetten[53] reported that with different samples of glycogen 45 to 51 per cent was degraded by beta-amylase. This was in agreement with the conversion limit of 47 per cent found for glycogen by Meyer and Fuld[63]. In 1957 Manners[64], in a review on glycogen structure presented data indicating that the beta-amylase degradation limits range from 40 to 54 per cent.

It is difficult to explain the difference in the range of values for the degree of branching of glycogens in this study (Table 1) compared with values in the literature. The care taken to avoid prolonged contact of glycogen with acid may have resulted in a preparation that was less degraded and thus increased the amount of glucose units available to beta-amylase. Larner[21] reported that if the glucose residues of the outer branches of the two glycogen samples studies were removed completely to form the limit dextrin, the glycogen would be degraded 61 per cent.

Ultracentrifuge studies: Table 2 shows the sedi-

mentation constants of glycogen calculated from the observations on the behavior of samples in the ultracentrifuge. The data show that the various specimens of glycogen sediment with a peak position which gave $s_{20,w}$ values in the range of 60 to 120 S. These S values would correspond to glycogens with molecular weights of 1×10^6 to 6×10^6. These data agree with the range of peak positions reported by Bridgman(57), Bell(65), and Polglase, Brown and Smith(66).

Bridgman(57) studied the behavior of liver glycogen in the ultracentrifuge and showed that little diffusion occurred. Bell also measured the diffusion constants of glycogen and showed them to be low. Therefore, the spreading of the sedimenting boundaries is due mainly to the polydispersity of the glycogen sample. The peak position would represent the most probable sedimentation value for a family of molecules of different sizes grouped about a mode(66). The photographic records of the sedimenting boundary of the various glycogens studied in the ultracentrifuge are shown in Plates 1, 2, and 3.

These results demonstrate that in the nonfasting rat, glycine feeding did not affect the size of the glycogen molecule as revealed by the sedimentation constants (76 S, 77 S, 85 S, and 79 S). This was also found to be true for the glycogens obtained from the 16 hour fasted animals except

Table 2

Sedimentation constants of pooled liver glycogens obtained from rats fed the glycine or the control diet and subjected to various lengths of fast.

	Glycogen extracted by hot alkali		Glycogen extracted by cold trichloroacetic acid	
Fast (hours)	Glycine-fed	Control-fed	Glycine-fed	Control-fed
0	76 S*	77 S	85 S	79 S
16	81	84	120	84
24	57	99	104	115

* The constants ($s_{20,w}$) are given in Svedberg units where S = 1 x 10^{-13}.

Each sedimentation constant represents the peak position of one glycogen sample isolated from pooled rat liver.

that the cold trichloroacetic acid-extracted glycogen obtained from the glycine-fed rats had a sedimentation constant of 120 S which represents of a larger molecule than the other samples (81 S and 84 S). The twenty-four hour fasted animals yielded glycogens with sedimentation constants of 57 S, 99 S, 104 S and 115 S. The data indicate that the average molecular weights of glycogen increased as the fasting progressed even though the level of liver glycogen was decreasing. This shift in the peak position of glycogen may represent the more rapid action of liver phosphorylase with the small glycogen molecules that might occur during a fast. This is discussed in more detail later.

Electrophoresis of glycogen: The electrophoresis of glycogen on glass fiber paper is a recently developed technique(51). In 1956 Bourne, Foster and Grant(68) using a borate buffer were able to separate various carbohydrates. By substituting 2 N sodium hydroxide for the borate buffer, Lewis and Smith(69) demonstrated that glycogens, assumed to be homogeneous, could be separated into two or more fractions by this technique. The movement of glycogen on the glass paper appears to depend upon the ionization of alcholic groups under the influence of the alkaline electrolyte.

Glycogens of different physical structure might be expected to migrate at different rates under the influence

Plate 1

The photographic records of the sedimenting boundary of hepatic glycogen obtained from unfasted rats fed the glycine or the control diet. The first exposure in each series was obtained after equilibrium had been obtained. Each exposure is separated by 480 seconds. The direction of migration of the peak position of glycogen from the center of rotation is toward the right.

Photograph Plate	Method of Extraction	Diet
1	Hot alkali	Glycine
2	Hot alkali	Control
3	Cold trichloroacetic acid	Glycine
4	Cold trichloroacetic acid	Control

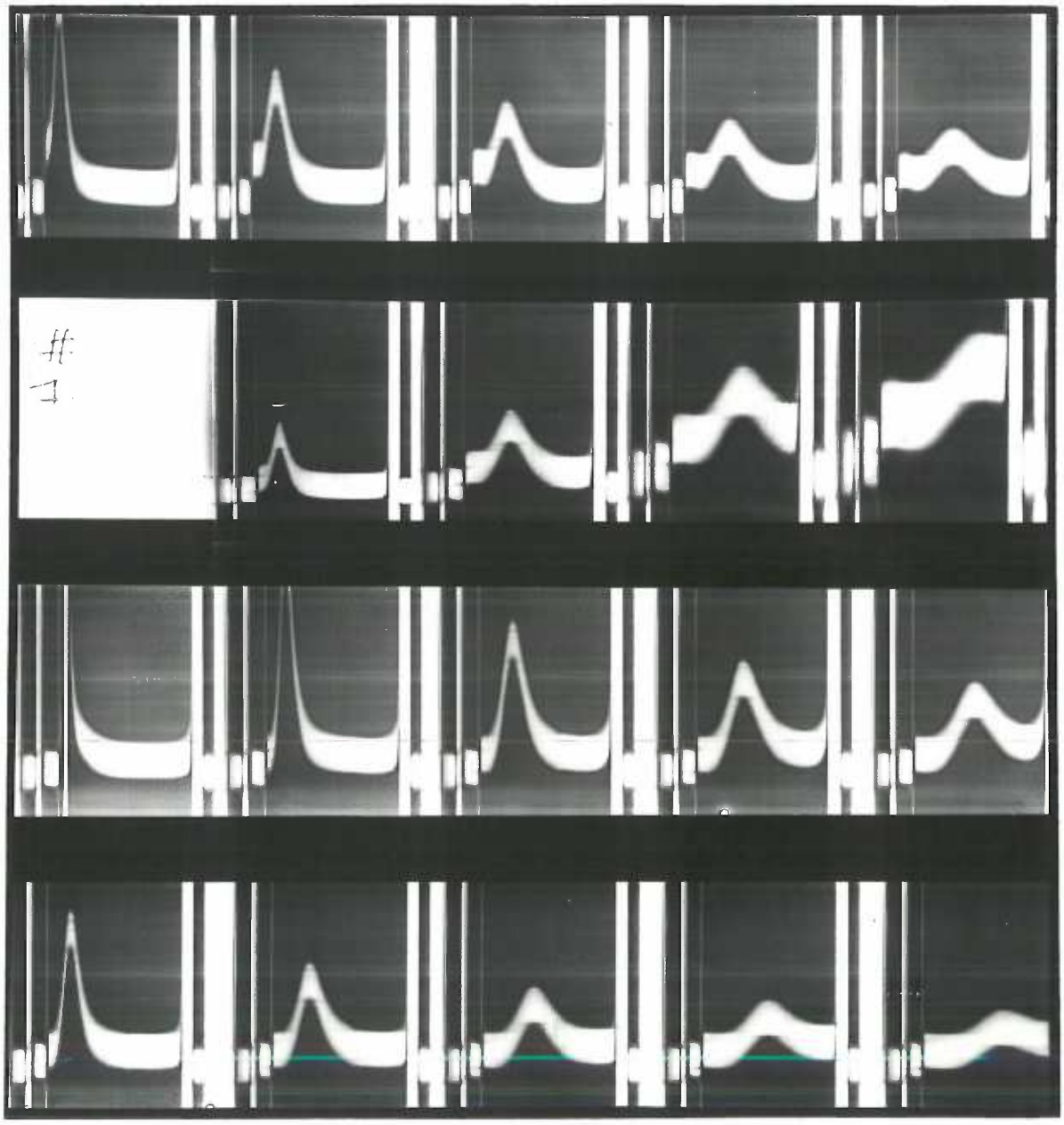


Plate 2

The photographic records of the sedimenting boundary of hepatic glycogen obtained from 16-hour fasted rats fed the glycine or the control diet. The first exposure in each series was obtained after equilibrium had been obtained. Each exposure is separated by 480 seconds. The direction of migration of the peak position of glycogen from the center of rotation is toward the right.

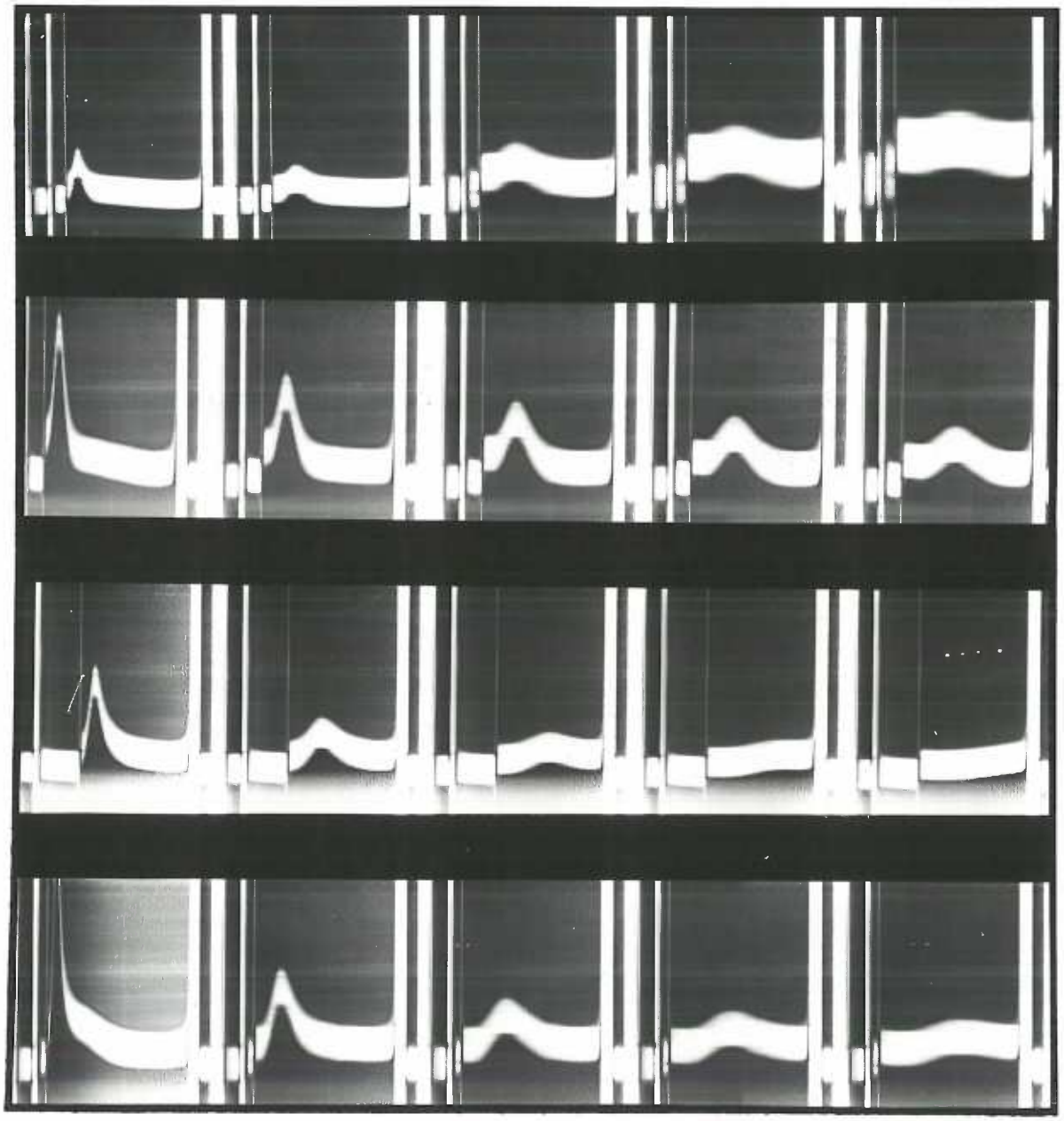
Photograph Plate	Method of Extraction	Diet
5	Hot alkali	Glycine
6	Hot alkali	Control
7	Cold trichloroacetic acid	Glycine
8	Cold trichloroacetic acid	Control

Fig.

Plate 3

The photographic records of the sedimenting boundary of hepatic glycogen obtained from 24-hour fasted rats fed the glycine or the control diet. The first exposure in each series was obtained after equilibrium had been obtained. Each exposure is separated by 480 seconds. The direction of migration of the peak position of glycogen from the center of rotation is toward the right.

Photograph Plate	Method of Extraction	Diet
9	Hot alkali	Glycine
10	Hot alkali	Control
11	Cold trichloroacetic acid	Glycine
12	Cold trichloroacetic acid	Control



of an electrical field. However, the glycogen samples obtained from glycine-fed and from control-fed rats by either method of isolation migrated at essentially the same rates. Also, none of the samples examined separated into more than one component under the experimental conditions employed. Such data indicate that glycine feeding does not alter the physical structure of liver glycogen, and that the two methods of isolation do not separate glycogens of grossly different structure.

It should be noted that the data represent single determinations. They are included simply to indicate the likelihood that this technique, as employed, may not be sufficiently sensitive to bring out slight differences in molecular weights and degree of branching if these exist in the samples studied.

Methods of isolation of glycogen from tissue: There has been controversy regarding the methods used to isolate glycogen from tissues since the days of Pflüger. This controversy had been renewed in intensity since Bloom and co-workers(45) reported that trichloroacetic acid extracted a constant amount of glycogen from tissue and that this quantity extracted reflected the physiologic status of the animal. Stetten and Stetten(70) isolated glycogen from the liver of one rat that had received C^{14}-sorbitol. The

Table 3

Electrophoresis of glycogen samples isolated from pooled livers of rats fed the glycine or the control diet and subjected to various lengths of fast.

	Glycogen extracted by hot alkali		Glycogen isolated by cold trichloroacetic acid	
Fast (hours)	Glycine-fed (cm. migration*)	Control-fed (cm. migration)	Glycine-fed (cm. migration)	Control-fed (cm. migration)
0	4.2	4.4	6.5	6.6
16	4.2	6.0	4.6	4.7
24	3.5	3.8	4.0	3.7

* Distance from point of origin.

glycogen of half of the liver was isolated by hot alkali; trichloroacetic acid was used to extract the glycogen from the remaining liver tissue. The two resulting glycogens were subjected to repeated purification steps until the yield and the specific activity of each was the same. The Stettens concluded that contamination of the total glycogen as determined by the method of Bloom was evident, and that the residual glycogen was an artifact.

Kahan(71) reported a method of isolation of total glycogen using trichloroacetic acid and barium hydroxide-zinc sulfate as protein precipitating agents. Carrol, Langley and Roe(72) also reported that trichloroacetic acid completely extracted the glycogen in liver and muscle. Tissue carbohydrate was isolated by the potassium hydroxide method and was dialyzed; the dialysate contained a material that reacted with anthrone. Dialysis of the tissue carbohydrate obtained by trichloroacetic acid did not yield an anthrone sensitive substance in the dialysate. Therefore, they concluded that the hot alkali extraction consisted of glycogen and a nonglycogen carbohydrate.

Carrol et al(72) also demonstrated that the amount of glycogen present in tissue after prolonged boiling in potassium hydroxide solution decreased and that there remained a high proportion of nonglycogen carbohydrate.

Stetten and Stetten[73] also studied the degradation of glycogen by hot alkali in atmospheres of oxygen and of nitrogen and by trichloroacetic acid at 23° C. and 0° C. It was demonstrated that potassium hydroxide caused a decrease in the molecular weight and that this decrease occurred more rapidly and more pronounced when the glycogen was heated in oxygen. Trichloroacetic acid also causes the molecular weight of glycogen to decrease; this decrease was less at 0° C. than at 23° C. Therefore, the Stettens chose to use cold trichloroacetic acid to extract glycogen. They stated that glycogens thus isolated represented native glycogen more closely than the other methods used for extraction.

Greenwood and coworkers[74] stated that 30 per cent potassium hydroxide at 100° C. degraded glycogen no more than boiling water and supported this with the determination of the sedimentation constants. They did demonstrate that heating glycogen in dilute alkali solution reduced the sedimentation constant from 86 to 57 S. As stated earlier, Bridgman[57] and Bell and Young[41] could demonstrate no change in the physical characteristics of glycogen when isolated by hot alkali or by trichloroacetic acid.

The "glycogen fractions" controversy: The quantitative aspect of glycogen fractions in tissues has been investigated by numerous investigators. Following the

initial report[45], Bloom and Knowlton extended their original studies to investigate the change in muscle glycogen fractions during stimulation and demonstrated that the decrease in total glycogen was accounted for entirely by the decrease in the trichloroacetic acid-soluble fraction[75]. Van Heijningen and Kemp[76] also studied the behavior of free and fixed glycogen in rat gastrocnemius muscle after stimulation of the sciatic nerve or after work. These workers noted that both fractions of glycogen decreased, but the decrease in the acid-soluble fraction was more pronounced. These workers believed that acid-insoluble glycogen was not an artifact due to the coating of precipitated protein around glycogen in tissue that did not permit it to be extracted. They showed that extraction of glycogen from tissue by a chelating agent that did not precipitate protein yielded the same values for the glycogen fractions as did trichloroacetic acid.

Time studies of the acid-soluble and acid-insoluble fractions of glycogen in the chick embryo were made by Szepsenwal and Partridge[77]. They demonstrated that with increasing age of the embryo, there was a characteristic pattern in the quantity of and in the fractions of glycogen present in the six tissues studied. The acid-soluble glycogen was only a small fraction of the total glycogen. The

increase in the acid-insoluble fraction of glycogen of striated muscle occurred during the last week of incubation, and this increase corresponded with the decrease and disappearance of the spontaneous activity exhibited by skeletal muscle. This raised the question whether or not the increase in the acid-insoluble fraction was one of the factors responsible for the change in the behavior of skeletal muscle, i.e., its transformation from spontaneously active muscle into adult muscle which responds only to stimuli.

Szent-Györgi[78] demonstrated that glycogen and myosin form an insoluble complex in vitro. This complex was dissolved upon the addition of adenosine triphosphate. Also, trichloroacetic acid extraction of the glycogen dissociated this complex in vitro. Szent-Györgi believed that this myosin-glycogen complex and its dissociation under physiologic conditions might play a role in the regulation of carbohydrate metabolism in muscle.

Leonard[79] examined the effect of hormones on the trichloroacetic acid-extractable glycogen in skeletal muscle in an attempt to find an explanation for his previous observations that cortisone and growth hormone inhibited the glycogenolytic action of epinephrine on certain skeletal muscles in the rat. He demonstrated that epinephrine decreased the total and acid-soluble fraction of rat

diaphragm and leg muscle. He was unable to demonstrate that growth hormone and cortisone prevented the glycolytic effect of epinephrine by converting the acid-soluble glycogen to the acid-insoluble form.

In 1956 Heijningen(80) reported that the increase in glycogen content of diaphragm as a response to insulin occurred in the acid-extractable fraction. The fixed glycogen remained constant.

It was also demonstrated by Pincus and coworkers(81) with the aid of labeled glucose, that insulin promoted an increase in the acid-soluble fraction of rat muscle and of liver. This fraction of muscle glycogen had a uniformly higher specific activity than the nonacid-extractable fraction. These changes were not as marked in the liver, probably because in the liver the acid-extractable glycogen of this tissue composes 85 per cent of the total glycogen (in the fed rat). It would be of interest to study the appearance of the labeled glucose in the acid-soluble fraction of glycogen with respect to time.

The effect of prolonged testosterone administration on acid-soluble and acid-insoluble glycogen in the rat levator ani muscle was studied by Meyer and Herschberger(82). They demonstrated that initially a striking increase in the glycogen concentration occurred. This increase was due

entirely to the trichloroacetic acid-soluble moiety while the acid-insoluble glycogen remained unchanged.

Merrick and Meyer(83) in 1954 repeated studies on the effect of anoxia in decreasing cardiac glycogen. The finding of interest was that acid-soluble glycogen decreased proportionally more than the total glycogen; this observation suggested to the authors the possibility that the absolute amount of residual glycogen may actually increase during the period of anoxia. The free glycogen might be converted to the fixed glycogen by which means it would be made available for the needs of the cell. In 1955, Meyer and coworkers(84) extended these observations by studying the changes in the glycogen fractions in heart homogenates. They demonstrated that in these preparations there was a decrease in both fractions when conditions favoring glycolysis existed. When the in vitro system favored glycogen synthesis, an increase in the acid-soluble fraction was noted in as soon as twenty minutes. Only after fifty minutes was a small increase in the acid-insoluble fraction found.

In studying the hormonal control of glycogen in the heart, Russell and Bloom(85) demonstrated that the cardiac glycogen of normal and adrenalectomized rats increased by 50 per cent during a 24 hour fast. If the animal was hypophysectomized, the cardiac glycogen concentration

decreased during this period of fasting. However, the administration of growth hormone permitted the normal increase in cardiac glycogen during fast. In the heart all the variations induced by the above experimental conditions occurred exclusively in the acid-extractable portion of the total glycogen. Continuing the experiments on the effect of growth hormone on cardiac glycogen in the rat, Adrounty and Russell[86] demonstrated that in both intact and hypophysectomized rats, growth hormone given during a fasting period increased the level of total and acid-soluble glycogen in the gastrocnemius and cardiac muscle. Following a 48 hour fast, the feeding of carbohydrate or protein, but not fat, to the intact rat resulted in an abrupt fall in cardiac glycogen, reflected by a decrease in the acid-extractable fraction.

Noble and Papageorge[87] studied the glycogen fractions of adrenal glands in the rat. The adrenal gland, like other tissues studied, contained a fraction of glycogen that was readily extracted with cold trichloroacetic acid. This fraction accounted for about one-half of the total glycogen. Fed rats had adrenal glycogen levels of 100 mgs. per cent while fasted rats showed an increase to 200 mgs. per cent. Seventy per cent of the adrenal glycogen was lost by fasted rats following feeding or the injection of epinephrine or

insulin. This disappearance of glycogen occurred in the fraction extracted by acid.

Russell and Bloom[88,89] extended their studies on the behavior of the extractable and residual glycogen in gastronemius muscle, heart, diaphragm, and liver of rats under a variety of conditions. They reported that the proportion of extractable glycogen varied in a constant manner with the amount of glycogen present. Other protein precipitating agents yielded the same amount of glycogen as trichloroacetic acid. If the tissues were extracted with water alone or with dilute sulfuric acid, more glycogen than usual was recovered. but if a protein precipitating agent was now added to this extract, the precipitation of proteins removed a constant quantity of the glycogen along with the protein.

Possible significance of glycogen fractions: The significance of this dichotomy of tissue glycogens has not yet been revealed. The studies by Bloom and by Russell indicate that the acid-soluble glycogen is free to participate in the metabolism of the cell. The fixed fraction may remain constant and act as a depot. Other workers, such as Merrick and Meyer, have attempted to assign to the acid-soluble glycogen the role of depot carbohydrate which must be converted into acid-insoluble glycogen if it is to become available to the cell.

Russell[90] reported on studies of the acid-soluble glycogen and the residual glycogen after the latter had been liberated by alkali digestion. She could find no difference in these glycogens using the measurements of degree of branching and behavior in the ultracentrifuge. Remarque[91] analyzed glycogen fractions according to Bloom and confirmed his findings. However, using the metaperiodate end group assay method he was able to demonstrate a shorter outer chain length for the residual glycogen. This difference in chain length was not revealed when trichloroacetic acid-extracted glycogen was compared with alkali extracted samples.

In 1934 Willstätter and Rhodewald[92] designated two fractions of liver glycogen as "lyo-glykogen" and "desmo-glykogen". The "lyo-glykogen" represented the extractable glycogen which formed up to 90 per cent of the total tissue glycogen although the ratio depended upon the nitritional state of the animal. They believed that the "desmo-glykogen" was bound to protein because it was partially set free by peptic digestion and was totally released by boiling in alkali. Other workers have demonstrated that glycogen can be adsorbed upon proteins and other colloids[93]. It has been demonstrated that glycogen can form a complex with myosin. These observations support the view that protein-binding of

glycogen may play a role in regulating the availability of glycogen to meet the requirements of cell activity.

Of interest to the problem of glycogen-protein complex are the observations made by Sutterland and Wosclait(94) during their preparation of phosphorylase. It was shown that glycogen accompanied liver phosphorylase during the purification procedures in the ratio of 20-30/1. In order to free the enzyme preparation of most of the glycogen the impure phosphorylase was adsorbed on a calcium phosphate gel and eluted. Furthermore, it was demonstrated that phosphorylase remained in the supernatant after centrifugation at 100,000 g. If glycogen was added to phosphorylase solution, the enzyme was no longer demonstrable in the supernatant following centrifugation; it was found in the sediment associated with glycogen. Is it possible that phosphorylase may be associated with its substrate in the cell? The regulation of phosphorylase action on glycogen may then be regulated by phosphorylase-rupturing enzyme or by hormones. This might help explain some of the observations made regarding differences in glycogen fractions resulting from various nutritional states or as a response to stresses in experimental animals. It also may be concerned with hormonal control of enzyme systems.

Lazarow(95) prepared a liver homogenate in normal

saline. By differential centrifugation he was able to separate glycogen from the solution in the form of particulate matter. This particulate was dispersed into smaller units when protein precipitating agents such as trichloroacetic acid were added. Moreover, he showed that his particulate form of glycogen contained small amounts of protein and theorized that this protein may play a role in the maintenance of glycogen in the particulate state in the cell.

Heterogeneity of glycogen: The differences in the amount of glycogen obtained by the various extraction techniques could be explained on the basis of solubility. Glycogen is known to be polymolecular. The smaller molecules of glycogen would be readily extracted by water, trichloroacetic acid etc. Meyer(96) postulated that "desmo-glykogen" represents a highly polymeric fraction of glycogen which is insoluble in acid and water and is soluble in alkali. The experiments of Meyer and Jeanloz(97) demonstrated that glycogen subjected to centrifugation or electrodialysis could be separated into three major divisions according to their degrees of solubility. Polglase et al(66) demonstrated that glycogen was polydisperse as did Bell(65) and Bridgman(57). Polglase also demonstrated that glycogen could be fractionated by centrifugation or by

treatment with various alcohol concentrations.

The most recent work on the molecular weights of glycogen as determined by light scattering technique yielded values of 10 x 10^6 to 159 x 10^6 for one sample of liver glycogen(50). The average molecular weights for trichloroacetic acid-extracted glycogens ranged from 11 x 10^6 to 80 x 10^6. Larner(39) determined that the distributions of molecular weights for muslce glycogen ranged from less than a million to over one hundred million with a broad maximum around 2.5 million.

Not only is glycogen heterogeneous with respect to molecular size but also with respect to its metabolic activity. Stetten and Stetten(53) examined the synthesis of new glycogen in liver and muscle from labeled glucose with beta-amylase and demonstrated that the radio-activity appeared initially in the outer branches of glycogen and with time the labeled glucose molecules were transferred to the inner aspects of the glycogen molecule, presumably due to the action of the branching enzyme. These observations were confirmed using the enzymatic degradation technique of glycogen developed in Cori's laboratory(98).

The study of the reactivity of glycogen molecules of various sizes towards phosphorylase was made both in vitro and in vivo(99). The results may be summarized as follows:

1) muscle phosphorylase reacts preferentially with the larger glycogen molecules; 2) when liver glycogen is treated with liver phosphorylase, the smallest molecules react most rapidly. Larner(39) supported the concept that muscle phosphorylase action takes place at the outer chains of the largest molecules of glycogen.

From the preceding discussion it should be evident that at the intermolecular level and at the intramolecular level, glycogen is metabolically inhomogeneous. The probable replacement of a glucose residue in glycogen does not occur in a random manner, but depends upon the tissue in which the glycogen is situated, the location of the glucose residue within the molecule and the size of this glycogen molecule(99). The glycogen molecule is in a continuous state of flux reflecting the relative rates of phosphorylase, branching and debranching enzyme activities. It also may undergo cleavage into large fractions due to the action of alpha-amylase.

The study of the effect of glycine on glycogen as revealed by measurements of whole populations of molecules did not aid in explaining the mechanism of the "glycine effect". It is proposed that further work glycogen as influenced by dietary glycine might well include 1) the study of the distribution of radioactive labeled glucose incorporated into glycogen, 2) the distribution of radioactivity

when glycogen is fractionated into populations of different molecular weights, 3) the study of radioactive glucose precursors such as glycine, serine phosphate or pyruvate, since the experiments of Marks and Feigelson[100] have indicated that the pentose phosphate pathway contributes significantly to the formation of glycogen glucose and that this is more apparent in the fasted than in the fed rat. Thus, if glycine feeding stimulates gluconeogenesis, the mechanism may be partly revealed by such studies.

SUMMARY

This study was undertaken to determine if alterates in the glycogen molecule might aid in explaining the "glycine effect". Glycogen was isolated from pooled rat livers. The rats had been fed the glycine or the control diet and subjected to various lengths of fast. Glycogen was isolated by the hot alkali method or by cold trichloroacetic acid. These isolated glycogens were carefully purified; precaution was taken to avoid the degradation of glycogen, especially when in contact with acid. Nitrogen content was used as a criterion of glycogen purity.

These purified glycogen samples were then examined in the following manner: 1) beta-amylase degradation, 2) sedimenting behavior in the ultracentrifuge, and 3) migration by electrophoresis. These methods failed to reveal any significant difference related to glycine feeding and failed to reveal difference between the glycogens isolated by the two methods employed.

The methods of isolation of glycogen are discussed along with the quantitative aspects of the glycogen fractions based on these two extraction methods. Also discussed is the present day concept of the metabolic inhomogeneity of glycogen on an intermolecular and an intramolecular level.

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