GLYCOGEN FRACTIONS OF RAT LIVER AND MUSCLE

by

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INTRODUCTION

Subsequent to the discovery of glycogen by Claude Bernard in 1857, various procedures have been devised for the removal of glycogen from tissue in order to study its nature. The early pioneers in this field noted that glycogen could not be extracted completely by hot or cold water and acids. Fränkel described in 1892 a method using trichloroacetic acid for extraction but commented that some glycogen still remained in the tissue residue. Loeschcke made a similar observation regarding water extraction. In 1906 Pflüger described an alkali method for glycogen extraction which, though modified by various workers, has remained to the present time the quantitative method of choice for the determination of glycogen in tissues. This method depends in the first place, upon the stability of glycogen in hot alkali, and secondly, upon the insolubility of glycogen in 60-70 per cent alcohol. However, the main drawback of Pflüger's method is the difficulty of freeing the glycogen precipitate from potassium hydroxide and protein degradation products. For this reason the qualitative preparation of glycogen used in studying the nature of the molecule includes various modifications of water and acid extraction techniques. Serious doubt has been cast on the stability of the glycogen molecule in alkali by later workers. However, Bell and Young[1] in 1934 noted that purified preparations of glycogen obtained by hot water extraction or by the Pflüger method do not exhibit any difference in their physical and chemical characteristics, such as specific rotation, ash content, reducing power, coloration with iodine, and polarimetric behavior during hydrolysis by acid.

Attention toward the quantitative aspect of water-extractable glycogen was directed by Chiao Tsai[2]. In 1933 this worker showed that in spite of repeated aqueous extractions there always remained a small quantity of glycogen in the residue of rabbit's liver. His observations were confirmed by Carruthers and Ling[3]; however, these investigators noted that this hot water-extractable fraction of glycogen was relatively constant irrespective of the variation in the total glycogen values.

During a study of the phosphate fractions in liver and muscle, Pinchot and Bloom[4] noticed that a significantly large fraction of the glycogen in both of these tissues was readily extractable by cold trichloroacetic acid and that in each case this readily extractable fraction seemed to make up a constant and reproducible proportion of the total glycogen. These observations led to a study by Bloom, Lewis, Schumpert, and Shen[5], in which this phenomenon was studied more critically in order to determine if this difference might be an artifact of the method; and, if not, to determine the behavior of these fractions in different physiological situations. These workers showed that the acid-extractable glycogen makes up a constant fraction of the total glycogen as determined by the hot alkali method. The trichloroacetic acid-extractable glycogen comprised 85 per cent of the total glycogen of the liver and 55 per cent of the total glycogen of the resting muscle of fed rats. When the rats were fasted, the acid-extractable glycogen disappeared more rapidly than the total glycogen; after 24 hours fasting the former fraction was nearly completely depleted. It was noted that upon feeding glucose to 24 hour fasted rats the acid-extractable fraction was quickly restored. However, the non-acid-extractable fraction was relatively unchanged

during the fasting and feeding periods. It should be remembered that Carruthers and Ling[3] also had reported that the non-water-extractable glycogen was relatively constant. Finally Bloom and associates showed that in muscle the decrease in glycogen following the administration of epinephrine occurred almost entirely in the acid-extractable fraction.

Hereafter, in this thesis, the acid-extractable glycogen will be called also the acid-soluble or the labile fraction, while acid-insoluble, stable, or residual fraction will refer to the non-acid-extractable glycogen.

The fact that various dietary procedures effect the non-fasting level of liver glycogen differently has been known for a long period of time. Mirski and coworkers[6] in 1938 undertook a study of the carbohydrate reserve in rats following various dietary regimens. These workers reported that unfasted animals fed a diet rich in carbohydrate had a higher liver glycogen reserve than did animals fed a diet rich in protein; but following a 24 hour fast or when subjected to cold for several hours, only traces of liver glycogen in the carbohydrate-fed animals could be demonstrated while in the protein-fed (meat or casein) animals a considerable amount of glycogen was present. In work experiment, the liver glycogen disappeared to a great extent in both series of animals, the protein-fed more than the carbohydrate-fed; however, after a rest of four to twenty-four hours without food, the animals which previously had consumed a high protein ration demonstrated glycogenesis with increased levels of liver glycogen, while the carbohydrate-fed animals showed a further decrease in liver glycogen. These workers called this ability of protein to maintain carbohydrate stores "the protein effect." In fasting

experiments the muscle glycogen behaved qualitatively the same as liver glycogen in animals fed these two types of diets. Regarding the amount of protein in the diet required to cause this effect, Mirski reported that 50 per cent protein was the minimum and 70 per cent protein was the optimum level. This "protein effect" is abolished by adrenalectomy. The demonstration of the "protein effect" on liver glycogen following a 24 hour fast was confirmed by Newburger and Brown[7] and by Guest[8].

When studying the effect of individual amino acids fed in conjunction with an otherwise satisfactory synthetic diet Todd and coworkers[9] showed that in animals fed a diet containing 10 per cent glycine (replacing an equal weight of the carbohydrate in the control diet) for 48 hours and then fasted 24 hours, the liver glycogen values were around one per cent, while in the control-fed animals the values were about 0.3 per cent. Glycine caused liver glycogen to behave in a similar pattern to that produced by a high protein diet. It is interesting to note that this "protein effect" caused by glycine can not be demonstrated in adrenalectomized animals.

Possible explanations of the "protein effect" of glycine include the stimulation of glycogenesis by glycine, or the possible glycostatic capacity of the amino acid, or a combination of the two. Further investigations were undertaken to elucidate the possible mechanism. Cunningham, Barnes and Todd[10] in 1947 studied the ability of rats prefed a glycine diet to withstand a large dose of insulin following a short fast in order to determine if this mechanism of action is a result of the glycostatic capacity of the amino acid. They observed that the drop in muscle and in liver glycogen as a result of insulin activity

was far greater in control-fed than in glycine-fed animals. Before and following insulin administration the blood sugar in the glycine-fed animals was significantly higher than in the control-fed rats. The recovery from insulin hypoglycemia was more rapid in the glycine-fed rats. It would do well to note that the control-fed animals received more carbohydrate than did the glycine-fed rats. This group of experiments ruled out the possibility of reduced glycogenolysis in the glycine-fed rats as a factor in accounting for the increased glycogen reserves.

Todd and Talman[11] in 1949 reported on studies concerned with the possibility that the extra carbohydrate content of the glycine-fed rats following a fast, might have arisen from the conversion of glycine stored as such or as a constituent of tissue proteins. Analyses of free and total glycine in blood, liver, muscle, intestine, and kidney were made. The data indicated that there is not sufficient extra glycine even if completely converted to carbohydrate to account for the differences in glycogen stores of glycine- and control-fed animals following fasting, or stress such as insulin administration.

Olsen, Hemingway, and Nier[12] fed fasted mice glycine labeled with the stable isotope of carbon in the carboxyl group. After 16 hours the liver glycogen had increased due to the metabolism of glycine, but they found a relatively small amount -- about one per cent -- of the fed isotope present in the glycogen. The rise in the liver glycogen was more than could be accounted for by the conversion of glycine to glycogen as measured by isotope excess method. Besides the direct conversion of glycine to glycogen probably through serine, glycine in some manner must promote the formation of glycogen from other body constituents. Dakin[13]

in 1921 believed that glycine "causes a disturbance in the normal equilibrium existing between the amino acids or peptides in the body with the result that other amino acids, capable of furnishing glucose are set free.

Mirski[6] reported that during a three day fasting experiment, his animals fed a high protein diet excreted larger amounts of nitrogen than did the animals on a normal protein diet during the first two days of the fast; by the third day the difference had decreased but still the protein-fed animals excreted more. Long, Katzin, and Fry[14] have shown that adrenalectomized animals are capable of normal carbohydrate metabolism while fed, but that a rapid disappearance of carbohydrate reserves and diminished nitrogen excretion occurs during fasting. Wells[15] has shown that adrenalectomized animals are capable of metabolizing dietary protein but are unable to metabolize endogenous protein well during fasting. Mirski[6] noted that in animals fed with an excess of fat and with the usual amount of protein in the diet, maintenance of the glycogen reserves was not observed during fasting. He concluded that increased protein catabolism stimulates glycogenesis so that in fasting and in other stresses glycogen is newly formed in the liver.

The most likely mechanism for the "protein effect" of glycine also seems to be increased glycogenesis, which apparently is related to increased adrenal cortical secretion.

PROBLEM

The glycogen fractions in liver and muscle were studied in order to determine if the "protein effect" of glycine may be attributed to an increase in the non-acid-extractable fraction which is known to be little effected by fasting and other stresses. Since the effect of glycine might be due to increased adrenal cortical activity, extracts of this gland were administered to control-fed, fasted animals to determine which glycogen fraction was effected.

EXPERIMENTAL

Animals: Two types of Sprague-Dawley rats from our own colony were used: young virgin females, about three months of age weighing approximately 200 to 250 grams, were used exclusively for muscle glycogen determinations while males, weighing 200 to 250 grams were employed in liver glycogen determinations. The weights within the experimental groups were matched closely as possible.

Rations: Colony rats were maintained on Purina Laboratory Chow. The compositions of the control ration and the glycine ration used in this and previous studies of the "protein effect" of glycine in this laboratory are shown below:

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

The compositions of the control and glycine diets are identical except that glycine was substituted for an equal weight of dextrin in the control diet. The large percentage of yeast satisfies the conditions that a high protein diet necessitates a high vitamin B intake for the most

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

efficient utilization of diet and for optimum food consumption. Several investigators recently have reported that small doses of cortisone administered to normal rats required increased vitamin B intake in order to maintain normal weight gains, hair growth, and prevent decrease in thymus weight[17, 18]. It can be inferred that possibly increased adrenal cortical activity would require increased vitamin intake.

The food intake of control- and glycine-fed animals was equalized. The animals were offered 12 to 15 grams of ration per 24 hours. Data are reported only from those rats which consumed the designated amount of the experimental ration.

Methods: The animals were anesthesized with Nembutal (0.5 ml.) intraperitoneally. When surgical anesthesia had been attained in those animals used for liver analyses, an abdominal incision was made, and the right and middle lobes of the liver were excised, the extra-hepatic tissue was cut away, and the liver blotted in order to remove as much blood as possible from the surface. In rats used for muscle studies, the left gastrocnemius muscle was freed of the plantaris and removed as a whole. Care was exercised so that the muscle underwent a minimal amount of contraction. Immediately following removal, the tissues were minced into previously weighed tubes containing 30 per cent potassium hydroxide or 10 per cent cold trichloroacetic acid. Less than one minute elapsed between the time the tissue was removed and immersed in the alkali or acid. Approximately one gram of tissue was used for the alkali determination of glycogen while 0.5 to 0.7 grams was used in the trichloroacetic acid method.

The Good, Kramer, Somogyi modification of the Pflüger method[19]

was used for the determination of total glycogen. Following the digestion of the sample in alkali on a boiling water bath, 1.1 to 1.2 volumes of 95 per cent ethanol was added to precipitate the glycogen. The sample was again immersed in the boiling water bath until the alcohol just began to boil in order to cause coarser particles of the glycogen precipitate to form. After centrifugation and draining off of the supernatant, the precipitate was washed with 95 per cent alcohol, centrifuged, and drained; the excess alcohol evaporated by placing the tube on a hot water bath for a few minutes.

Determination of the acid-extractable glycogen was accomplished by adapting the method described by Bloom and coworkers[5]. In this procedure the weighed tissue in trichloroacetic acid was ground to a fine suspension by a Potter-Elvehjem homogenizer. The contents of the tube were transferred quantitatively to a 40 ml. centrifuge tube, and the grinder was washed with trichloroacetic acid so that the final volume of acid used was 15 ml. After centrifugation for 10 minutes at 1200-1500 r.p.m., the supernatant solution was decanted and mixed with an equal volume of cold 95 per cent ethanol; it was left in the cold room for at least 24 hours. Then the white flocculent precipitate was separated by centrifugation and washed once with 95 per cent ethanol. Bloom reported that when the protein precipitated by trichloroacetic acid was reground with acid, less than 0.01 per cent glycogen was recovered from the supernatant of liver residue while no glycogen was found from the re-extracted protein precipitate of muscle.

To each of the glycogen precipitates 3 ml. of 1 N sulfuric acid was added, and the samples hydrolyzed for three hours on a boiling water bath.

Following hydrolysis the acid solutions were neutralized to the phenol red endpoint with dilute potassium hydroxide; these neutralized solutions were diluted to such a volume that 5 ml. contained approximately one milligram of glucose which was determined by the Shaffer-Hartmann method.

Plan of experiment: In the afternoon rats were removed from the colony, weighed and placed in individual cages which were equipped with screen floors to prevent coprophagy. Water was offered ad libitum; food was offered in large cups which were designed to reduce spillage and contamination of food. Each animal received 6-8 grams of the control ration for the first night in order to accustom the rat to a powdered synthetic diet and better to control the amount of ration eaten during the succeeding two days.

In the morning of the second day, the animal was offered 12 to 15 grams of the glycine ration or continued on this amount of the control ration. On the morning of the third day, two-thirds of the daily ration was given; the remaining one-third was not offered until the last two hours of the feeding period. This procedure was employed in order to regulate the length of fasting. At this point the animal was fasted for various lengths of time and then removed from the cage, weighed and sacrificed for the glycogen determinations. In general, the animals studied at 0 hour, 24 hour, and 48 hour fasts were killed between 9 and 12 a.m. Those animals fasted 16 hours were fed twice a day, at 9 a.m. and 9 p.m., in order to eliminate the possibility that all food consumption might occur at night; these animals were killed at 1 p.m.

RESULTS

Tables 1 and 2 present a tabulation of the amounts of glycogen extracted by hot alkali or by trichloroacetic acid from liver tissue of rats fed the control or the glycine ration, and then subjected to various lengths of fasting. In the Tables and throughout the discussion, glycogen is expressed as grams of glucose per 100 grams of wet tissue. The values of total glycogen resulting from dietary procedures closely approach the values found previously in this laboratory. The difference shown between the control-fed and the glycine-fed, unfasted animals does not appear to be significant as demonstrated by the standard error of the mean. The glycogen levels for the glycine-fed animals and for the control-fed animals are 4.77 per cent and 3.94 per cent respectively. These values compare well with the level of 4.55 per cent found in a series of control-fed, unfasted animals studied in another connection. In both series of unfasted animals, the non-acid-extractable fraction comprised 10-15 per cent of the total glycogen.

Following a 24 hour fast, the glycine-fed animals maintained their carbohydrate stores at a level almost three times as high as the control-fed animals. This increased level was found to be for the most part in the acid-extractable fraction. Compare, for instance, the values 0.89 per cent and 0.24 per cent for the two fractions.

In the case of the control-fed animals the acid-soluble glycogen was rapidly depleted during fasting. The non-acid-soluble glycogen was slightly decreased, and by the end of the 24 hour fast it comprised 45 per cent of the total glycogen.

The liver glycogen of the 16 hour fasted, control-fed animals were

studied next. This length of fast was chosen because previous work had shown that the total glycogen values of liver in such animals was about one per cent which is comparable to the glycogen level of glycine-fed rats following a 24 hour fast. It is interesting to note that the stable glycogen comprised 22 per cent of the total glycogen in this series of 16 hour fasted, control-fed rats while in the 24 hour fasted, glycine-fed rats it amounted to 20.5 per cent (note the last column in Table 1 and Table 2). Animals fed glycine and fasted 16 hours showed total glycogen levels around two per cent, and the stable glycogen comprised approximately 17 per cent of this.

In addition, liver glycogen values were collected for 32 and 48 hour fasting animals. The control-fed animals seem to reach a minimum liver glycogen content after about 32 hours of fast; this value then begins to increase gradually as shown by the 48 hour values. These data agree well with the values reported by other workers[20]. On the other hand, the glycine-fed rats show a continued decrease of glycogen at both of these periods of fasting.

Since the mechanism of the "protein effect" of glycine is thought at this time to be the result of increased adrenal cortical activity, it was of interest to investigate which fraction of liver glycogen would be influenced by the administration of adrenal cortical extract during a 24 hour fasting period. Adrenal cortical extract administered to a normal fasting animal will cause the liver glycogen reserve to be greater than that of an animal not receiving the extract[14]. This represents the known stimulation of glycogenesis by adrenal hormones. Rats on the control diet were fasted for 24 hours. Upjohn adrenal cortical extract

was administered subcutaneously in 1 ml. doses at 20, 16, 12, 8, 6, and 4 hours before the end of the fasting period. It can be seen in Table 1 that adrenal cortical hormones cause an increase in the acid-extractable fraction. It is interesting to note that adrenal cortical extract resulted in a liver glycogen reserve similar in composition to that found in the 24 hour fasted, glycine-fed animal.

From Tables 1 and 2 these observations should be noted: (1) the acid-extractable glycogen is rapidly depleted during fasting in the control-fed animals and is diminished slowly in the glycine-fed animals; (2) the stable fraction is relatively little changed by feeding either of the two diets used, but shows a gradual decline during fasting. Graphs 1 and 2 illustrate these salient points.

The question then arose: can the glycogen which is freed by treatment with hot alkali be extracted completely by trichloroacetic acid? Two samples of minced liver from each of a series of unfasted, control-fed animals were studied. One of the samples was treated as previously described for total glycogen. The other sample was digested in alkali for three hours in a boiling water bath, and the glycogen precipitated with ethanol. This was centrifuged, washed with ethanol, centrifuged again, and the remaining traces of alcohol removed. Successive extractions using 5, 4, 3, and 3 ml. of cold trichloroacetic acid were made of the glycogen precipitate. To the combined extractions 15 ml. of 95 per cent ethanol were added to precipitate the glycogen. Glycogen was determined as previously described. The resulting residue was subjected to acid hydrolysis in order to determine if any glycogen remained. On one occasion petroleum ether was used to wash the residual precipitate with the

Table 1

Total and Acid-Extractable Liver Glycogen of Fed and Fasted Rats Previously Fed the Control Diet

Hours fasted	Total % wet weight	Acid-extractable % wet weight	Non-acid-extractable	
			% wet weight	% of total
0	3.94 ± 0.35* (6)	3.54 ± 0.32*	0.40 ± 0.05*	10.2 ± 0.6
16	1.28 ± 0.10 (5)	0.99 ± 0.09	0.29 ± 0.05	22.2 ± 1.8
24	0.38 ± 0.03 (9)	0.21 ± 0.03	0.17 ± 0.01	45.0 ± 4.5
32	0.15 ± 0.01 (4)	0.02 ± 0.01	0.13 ± 0.01	85.1 ± 3.2
48	0.32 ± 0.02 (4)	0.18 ± 0.03	0.14 ± 0.02	44.6 ± 8.0
24 plus Adrenal cortical extract	1.25 ± 0.26 (4)	1.02 ± 0.25	0.23 ± 0.02	18.0 ± 2.9

* Standard error of mean.

Number of animals used in each study included in parentheses.

Table 2

Total and Acid-Extractable Liver Glycogen of Fed and Fasted Rats Previously Fed the Glycine Diet

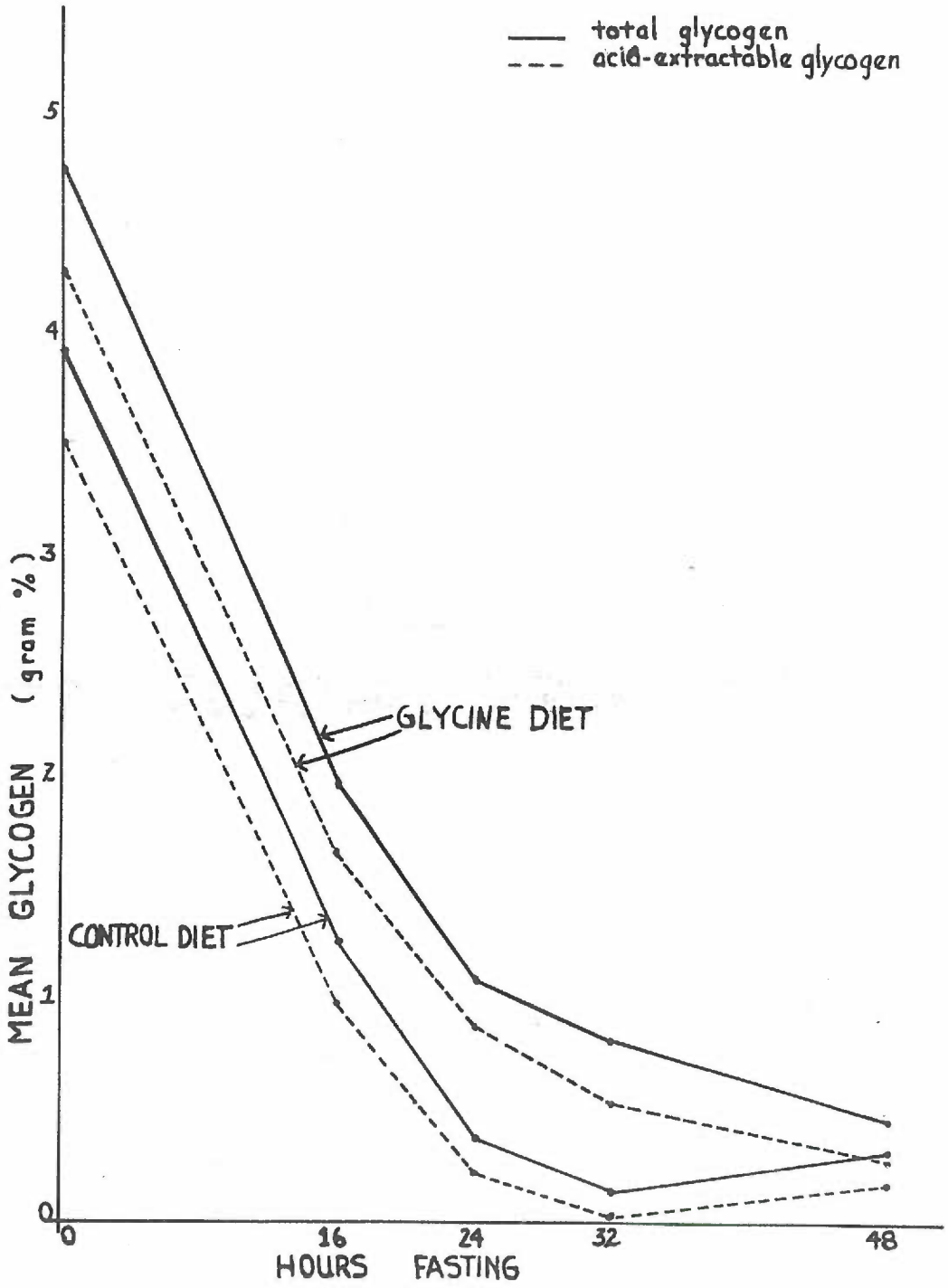
Hours fasted	Total % wet weight	Acid-extractable % wet weight	Non-acid-extractable	
			% wet weight	% of total
0	4.77 ± 0.40* (6)	4.31 ± 0.40*	0.46 ± 0.06*	10.0 ± 0.9*
16	2.00 ± 0.14 (7)	1.67 ± 0.14	0.33 ± 0.05	17.1 ± 2.6
24	1.13 ± 0.06 (6)	0.89 ± 0.06	0.24 ± 0.02	21.1 ± 1.7
32	0.86 ± 0.23 (4)	0.53 ± 0.13	0.33 ± 0.10	37.4 ± 2.6
48	0.48 ± 0.15 (4)	0.30 ± 0.08	0.18 ± 0.06	37.2 ± 2.0

* Standard error of mean.

Number of animals used in each study included in parentheses.

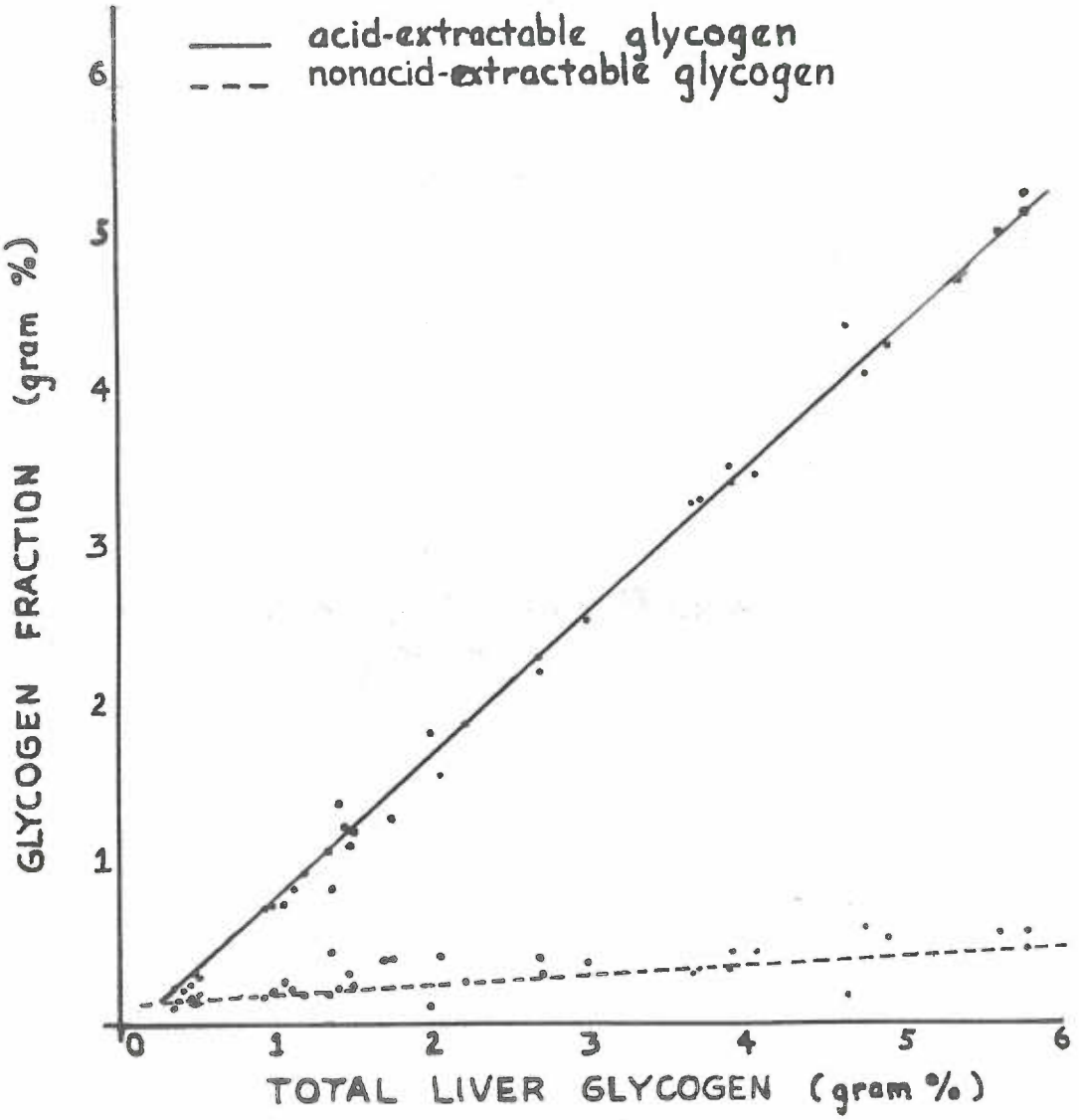
Graph 1

Behavior of the Liver Glycogen Fractions during Fasting
in Control-Fed and Glycine-Fed Rats


total glycogen
acid-extractable glycogen
MEAN GLYCOGEN (gram %)
5
4
3
2
1
0
GLYCINE DIET
CONTROL DIET
0
16
24
32
48
HOURS FASTING

Graph 2

Liver Glycogen Fractions versus Total Glycogen


acid-extractable glycogen
nonacid-extractable glycogen
GLYCOGEN FRACTION (gram %)
TOTAL LIVER GLYCOGEN (gram %)
0
1
2
3
4
5
6

result that a considerable portion of this residue dissolved, illustrating that some lipid had been precipitated with the glycogen.

The results of this study are shown in Table 3. The values for total glycogen are not statistically different from those for glycogen extracted by trichloroacetic acid following alkali digestion of tissue. In other words, no glycogen could be demonstrated in the residue following acid extraction. If, however, the tissue was not hydrolyzed with alkali for several hours, the residue showed traces of glycogen following the extraction procedure. The result of this study lends support to the thesis that alkali treatment frees glycogen from other tissue constituents and allows it to be completely extracted by acid. It should be clear that these findings do not indicate inaccuracy of the method used for total glycogen, since in that procedure the glycogen precipitated along with small amounts of protein and other impurities is subjected to a three hour hydrolysis with 1 N sulfuric acid. This converts all the glycogen — free and combined — to glucose.

In Table 4 are the results of studies made on the total and acid-extractable muscle glycogen of fed and fasting rats previously fed control or glycine diets. These data indicate that the control-fed animals have a slightly higher muscle glycogen content (0.76 per cent) than the glycine-fed rats (0.60 per cent). The stable glycogen comprised 40 to 45 per cent of the total in both groups. Following a 24 hour fast the acid-soluble fraction diminished to a somewhat greater extent in the control-fed rats. This decline in the labile fraction is not nearly as pronounced in muscle glycogen as in liver glycogen under similar experimental conditions. Mirski[6] also reported similar changes in total muscle glycogen in fasted animals previously fed a high protein diet.

Table 3

Total and Acid-Extractable Liver Glycogen
Following Alkali Digestion

Total (KOH digestion) % wet weight	Acid-extractable following alkali digestion % wet weight	Residue % wet weight
4.70	4.90	0
4.23	4.19	0
4.74	4.88	0
3.94	3.78	0
4.97	5.17	0
4.18	4.14	0
5.15	5.27	0
Mean: 4.55	4.62	0

Each horizontal row of figures represents data from aliquots of one liver.

Table 4

Total and Acid-Extractable Muscle Glycogen of Fed and Fasted Rats Previously Fed Control or Glycine Diet

Hours fasted	Control diet				Glycine diet			
	Total % wet wt.	Acid-extractable % wet wt.	Non-acid-extractable		Total % wet wt.	Acid-extractable % wet wt.	Non-acid-extractable	
			% wet wt.	% total			% wet wt.	% total
0	0.77±0.03* (7)	0.43±0.04*	0.34±0.03*	44.4±3.7*	0.60±0.03 (6)	0.36±0.03	0.24±0.02	40.5±2.3
24	0.44±0.02 (7)	0.21±0.01	0.23±0.01	52.6±1.8	0.50±0.04 (5)	0.25±0.03	0.25±0.02	51.0±2.3

* Standard error of mean.

Number of animals used in each study included in parentheses.

DISCUSSION

The work reported here and that of Bloom and associates[6] support the thesis that the fractions of glycogen form characteristic proportions of the two tissues studied — the acid-extractable fraction forms approximately 85-90 per cent of the total liver glycogen and about 55-60 per cent of the total muscle glycogen in the unfasted rat. These two fractions of glycogen behave differently when the physiological state of the animal is altered. The data of this study show that dietary glycine effects the acid-extractable glycogen and not the non-acid-extractable fraction, which is in general, only slightly altered by feeding and fasting procedures. The "protein effect" of glycine cannot be explained on the basis that glycine feeding brings about an increase in the concentration of the glycogen fraction which is relatively stable.

Tsai[21] reported in 1937 that the "combined glycogen" content in the liver of cats was only slightly effected by 2-3 days starvation with or without glucose feeding or by previous feeding on a high protein diet. It should be of interest to remark in passing that Donhoffer and Macleod[22] postulated two forms of liver glycogen existing in distinct physiological states: one being a storage form derived from preformed glucose; and the other, a glycogenic form synthesized from protein, lactic acid, and other glucose precursors, and not generally stored. Later studies on carbohydrate metabolism and glycogen synthesis do not support this hypothesis.

This difference in behavior exhibited by these glycogen fractions toward varying physiological conditions might be attributed to differences in the state of tissue glycogen. Often the question has been raised

whether glycogen in tissue is combined with protein. Studies by Willstätter and Rohdwald[23] in 1934 designated the water-extractable fraction of liver glycogen as "lyo-glykogen" and the unextractable, "desmo-glykogen." They reported that the relative proportions of these two forms of glycogen depended on the nutritional state of the animal: in the well fed animal the lyo-glycogen may amount to 90 per cent of the total, in the starved animal this fraction may be 10 to 60 per cent of the total. They believed that the desmo-glycogen is bound with protein because it can be set free in part by peptic digestion and totally released by boiling the tissue residue in alkali. In vitro studies lend support to a glycogen-protein complex. Przylecki and Wojcek[24] in 1928 demonstrated that glycogen can be adsorbed upon proteins and other colloids, but this glycogen can be quantitatively eluted with water. When glycogen or starch is added to a weakly alkaline solution of myosin, the opalescence is intensified, indicating that the particles have become coarser. When this solution is neutralized, the precipitated glycogen carries down a considerable amount of protein with it. Przylecki in a series of papers published during 1934-35 postulated that myosin and starch form a compound whose composition is approximately 20 per cent starch[25, 26]. No constant value was stated for glycogen. In studies conducted by Russian workers, Rosenfeld and Plychavskaja, glycogen treated with phosphorylase loses its ability to react with proteins[27]. Phosphorylase incompletely cleaves glucose residues from terminal branches of the glycogen molecule. They stated that the formation of glycogen-protein complexes depends on the terminal non-aldehydic groups. Pflüger attributed the inability to remove glycogen completely with hot water to the partial surrounding of glycogen by coagulated protein.

The nature of the glycogen molecule eliminates the possibility of primary valence bonds existing between glycogen and protein. Glycogen is composed of glucose units linked primarily by alpha-1-4 glucosidic linkages. By means of methylation studies of glycogen Haworth and associates[28] have determined to what extent the glycogen molecule is branched, and that this branching is primarily of 1-6 type. In their studies they found two types of glycogen, one consisting of 12 glucose units per branch point and the other, 18 glucose units. They proposed for glycogen a laminated type of structure in which each branch arises from the preceding branch. Staudinger and Husemann offered a comblike structure for glycogen in which each branch arose off the main chain. Meyer and coworkers[29] proposed a multibranched, tree-like structure which consisted of three to five glucose units between branch points and six to seven units in the outer branches. This theory has received the most support. Mathematically Myrbäck[30] proposed a theoretical form of glycogen which closely resembled that structure based on chemical evidence proposed by Meyer. Recently, Larner and coworkers[31] used an enzymatic degradation technique to study the glycogen molecule. Their results supported the tree-like structure. Larner stated that glycogen probably is a group of compounds containing a spectrum of varying branch lengths. The glycogen isolated may contain primarily short or long branches whose length depends on the previous history of the animal studied.

Studies by Meyer and associates support the theory that glycogen itself occurs in different degrees of polymerization with different solubilities. Small glycogen molecules are easily extracted from tissue while desmo-glycogen is in reality a high polymeric fraction of glycogen which

is insoluble in acid and water and soluble in alkali. This view stems from experiments by Meyer and Jeanloz[32] in which they subjected purified glycogen to centrifugation or electrodialysis and separated it into three major divisions depending upon their degrees of solubility. One group was insoluble in water even though the glycogen was freed from protein. Meyer is in agreement with Pflüger when he states that there are no grounds for assuming a union between glycogen and protein in tissue, for the variable solubilities of glycogen would be sufficient in itself to explain the varying solubilities in alkali, acid or water. However, the possibility of a glycogen-protein complex cannot be entirely excluded on the basis of the available evidence.

Unpublished work of van der Wyck shows that the molecular weight of glycogen amounts to 1 to 10 million[29]. He believes that glycogen molecules may be mechanically entangled to form macro molecules. Lazarow[33] is of the opinion that extraction, whether by alkali, acid, or water, changes the glycogen status of tissue. He has isolated a submicroscopic complex of glycogen by means of a high speed centrifuge. Further investigation of this "particulate glycogen" showed that it contained seven to eight per cent water after thorough drying which lends support to the concept of storage of water within the molecule. It is likely that hydrogen bonding can occur due to the presence of unshared electrons in the hydroxyl groups of the glucose units. If glycogen exists in a helical structure as proposed by Schlamowitz[34], water molecules may be trapped within the core. This may explain the difficulty in freeing glycogen from the last traces of water. Separation of the particulate glycogen from tissue seems to support the hypothesis that bound glycogen does exist in tissue because

this complex contains approximately 1 per cent protein. It is interesting to note that these particles are stable at 37° C., but can be dissociated into smaller units by heat, trichloroacetic acid, or potassium hydroxide, the agents used in glycogen extraction techniques. No reports of a glycogen free of phosphorus and of nitrogen have been found in the literature. This fact is in agreement with Lazarow's feeling that some protein always accompanies the most highly purified glycogen.

The above discussion indicates that the problem of whether glycogen exists in a free or in a bound form in tissues is not settled satisfactorily. Evidence presented in this thesis is entirely in favor of the view that at least part of the glycogen of liver and of muscle exists in a bound form. This is based upon the observation that glycogen can be extracted completely from tissue with trichloroacetic acid only after the tissue has been digested for about three hours with alkali. No evidence of glycogen remaining in the residue can then be found. The procedure of digestion in hot alkali may in some manner break the molecular bonding between glycogen and protein, possibly untangling the particulate glycogen and dissociating it into smaller units. Certainly finer techniques have to be developed before a solution to this problem can be obtained.

One might theorize that a particular type of cellular protein binds glycogen and that during fasting this protein is depleted. Such a depletion would result in the conversion of stable glycogen to labile or acid-extractable glycogen.

Previous studies of liver glycogen during fasting reveal that glycogen decreases rapidly during the first 24 hours of fasting and reaches a low point around 32 hours following removal of food; then it begins to

increase again[20]. This increase has been attributed to stimulation of glycogenesis. The total glycogen level of the control-fed animals in this study show the same rapid drop and lowest value at approximately 32 hours following their last meal and then a gradual increase. The question has been raised whether this is a true increase in total glycogen because the values are expressed as grams of glycogen per hundred grams wet weight of liver tissue. Since the weight of the rat liver decreases during fasting, this increase might be an artifact and express only a relative increase while there is actually a real decrease in the total amount of glycogen present. The behavior of the acid-extractable fraction may help to answer the question. It will be seen in graph 1 that the labile glycogen rapidly diminished during the first 24 hours of fasting, reached a low point about 32 hours and then began to increase again, paralleling the behavior of the total glycogen during the fast. This increase in the acid-soluble fraction following the 32 hour fast would support increased glycogenesis, since the liver weight is still decreasing.

Since the liver glycogen level in control-fed rats began to increase after a fast period of about 32 hours, it may well be that the very low level of labile glycogen (around 0.02 per cent) is responsible for triggering some mechanism resulting in adrenal cortical stimulation. In agreement with such a view is the work of Deane and coworkers [35] who studied some of the factors influencing glycogen synthesis by rat liver slices in vitro. One observation made in this investigation was that the liver slices initially must have low glycogen values if synthesis was to occur.

However, the liver glycogen levels of the glycine-fed animals did not show the same behavior during fasting as observed in the control-fed

rats. After 24 hours of fasting it was three times higher than the liver glycogen level of the control-fed rats. By the end of 48 hours of fasting, the liver glycogen had not reached the low level obtained in the control-fed animals earlier. Glycine fed at the level of 10 per cent in the diet is unphysiological and may act as a humoral stimulus for increased adrenal cortical activity. It will be of interest to study longer fasting periods with rats previously fed the glycine ration to discover if the glycogen level falls to a minimum value and begins to show a secondary stimulus of increased glycogenesis.

Actually, since glycogenesis results in an additional glucose supply to the body, one might expect the labile glycogen fraction to increase when this process is active. As mentioned previously, feeding glucose to a fasting animal does increase this fraction of liver glycogen.

In addition to this, the data indicate that after the administration of adrenal cortical hormone to control-fed animals, the distribution of the two glycogen fractions is altogether similar to the distribution in the 24 hour fasted, glycine-fed rats. This fact may be interpreted as resulting from increased adrenal cortical activity during the stress of fasting in the animals fed the glycine diet.

The observation that the acid-extractable fraction of liver glycogen in glycine-fed animals is more efficiently maintained than in control-fed animals during fasting supports the theory that glycogenesis is stimulated as a result of glycine feeding.

SUMMARY

A study was made to determine which fraction of liver glycogen is increased as a result of the "protein effect" of fed glycine in the rat. It was found that feeding glycine influences the acid-extractable glycogen, the fraction which shows great variability in amount and rapidity of depletion and recovery during periods of fasting and feeding. The effect of adrenal cortical extract on glycogen reserves during a fast is also primarily concerned with the maintenance of the acid-extractable fraction. These observations lend further support to the theory that dietary glycine stimulates the glycogenic processes as a result of increased adrenal cortical activity. The glycogen fractions of muscle have also been studied during feeding and 24 hour fasting experiments. The response was similar to that found in the liver glycogen studies.

The significance of these two fractions of glycogen is discussed in terms of present theories, one of which states that glycogen does exist in a bound form which is not extracted easily from tissue, and the second which attributes the partial insolubility to highly polymeric forms of glycogen.

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