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STUDIORUM PROGRESSUS

Iodine Adsorption Characteristics of Newly-Formed Rat Liver Glycogen

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In an earlier study on livers of diabetic rats injected with insulin[2], attempts were made to measure glycogen by the iodine adsorption method of VAN WAGTENDONK[3]. Values as high as 26 per cent of the wet weight of the tissue were obtained by this method. These values for glycogen failed to agree with those obtained by the classical method in which the glycogen was hydrolysed with acid and the resulting glucose estimated by its reducing value. It was this discrepancy that led us to study iodine adsorption by glycogen under experimental conditions in which glycogen is rapidly deposited.

Experimental. Treatment of Animals. Normal Rats. Rats of the Long-Evans strain, weighing 200–300 gm, were used. These animals were maintained on an adequate stock diet until three days before they were sacrificed. During those 3 days they were fed a high carbohydrate diet containing 58% glucose, 22% casein, 6% salt mixture, and 14% celluflour-vitamin mixture. The salt and celluflour-vitamin mixtures have been described elsewhere[2].

Diabetic Rats.–Male rats of the Long-Evans strain were made diabetic by an intravenous injection of 45 mg per kilo body weight of alloxan monohydrate (EASTMAN). Records of food and water consumption, urine excretion, weight changes, and glucose excretion in the urine were kept for a period of one month before the start of the experiment. During that time the animals manifested fasting blood glucose levels of 200 mg per cent or higher.

The rats that were to receive insulin were fed the high carbohydrate diet for several days before the start of the insulin injections, and were continued on that diet during the injection periods. The insulin treatments are described in Table 1. Rats that received no insulin were fed the high carbohydrate diet for 3 days and were then sacrificed.

Rats That Were First Fasted and Then Intubated with Glucose.–Rats weighing 200–250 gm were maintained on the stock diet until the start of the fasting period, which lasted for 18 h. At the end of the 18 h, each rat was intubated with 3 gm of glucose in a 50% solution, and was allowed access to the high carbohydrate diet until sacrificed. Pairs of these rats were sacrificed at 3, 6, 9, 12, 18, and 24 h after intubation of the glucose.

Methods. Hydrolysis of Tissue.–The rats were sacrificed by cervical fracture. Their livers were excised, placed in iced saline, and then sliced freehand as rapidly as possible. Quadruplicate samples of about 500 mg each were removed, blotted, and weighed. The samples were placed in 6 ml of hot 30% (w/v) KOH and digested for 2 h at 100°. The mixture was cooled, transferred to volumetric flasks, and made to volume with the 30% KOH. This digest was filtered through Whatman No. 41 filter paper to remove soaps and other insoluble components. Suitable aliquots of the filtrate were taken for the analysis of glycogen as described below.

Glycogen Analyses. Titrimetric Method.–0·5 ml of a saturated solution of Na_2SO_4 was added to a suitable aliquot of the filtrate, and the resulting solution was mixed with 1·2 volumes of ethyl alcohol[1]. The mixture was stored at 0° for 3 h, then centrifuged, and the precipitate thus obtained was dissolved in 2 ml of 1N HCl. The glycogen was hydrolysed for 2 h at 100°. The hydrolysate was neutralized with NaOH and Na_2CO_3, clarified with $Zn(OH)_2$[2], and centrifuged. Glucose was determined in the supernatant by the ferricyanide-ceric sulfate method[3]. The titration values were referred to a glucose standard, and are expressed as per cent glycogen (as glucose) of the wet weight of the liver.

Determination of Glycogen by Iodine Adsorption.– The method used was essentially that of VAN WAGTENDONK *et al.*[4]. The optical density was determined at 500 mμ, with all solutions carefully maintained at exactly the same temperature. A Model DU Beckman Spectrophotometer was used for the measurement of the iodine complex. The optical density of the iodine-glycogen complex was found to obey BEER's Law only when the electrolyte concentration was held rigidly constant. Each experimental group of rats included a normal animal whose liver glycogen served as a standard for the calibration of optical density of a given glycogen sample in terms of glucose.

Preparation of Purified Glycogen.–Rats that had been treated as described above were sacrificed by cervical fracture. Their livers were quickly removed, weighed, and transferred to two volumes of hot 30% NaOH. Digestion was carried out for 2 h at 100°C in a nitrogen atmosphere. Glycogen was isolated as outlined by SOMOGYI[5], then dried *in vacuo* at 60° for at least 24 h. Samples of these purified glycogens were weighed and then dissolved in water. Optical density readings were carried out at 25° on a solution containing the following per milliliter: 0·1 mg glycogen, 5 mg KCl, 3 mg KI, and 0·5 mg I_2. This solution, minus glycogen, was used as a blank against which samples were read. The readings were then referred to a sample of normal glycogen that had been purified in the same manner. Light scattering at this glycogen concentration is negligible.

Results.–Each liver was digested with KOH, as described above, and separate aliquots of each digest were taken for the analyses of glycogen by the two methods under consideration. Table I shows results obtained with a series of livers excised from 15 diabetic rats (12 of which had been under the influence of insulin for varying periods before they were sacrificed). The values obtained by the two methods were in fair agreement in the case of the untreated, diabetic rat livers. But a discrepancy—in some cases quite pronounced—was observed when glycogen samples obtained from insulin-treated, diabetic rats were analysed by the two methods.

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Table I.–A Comparison of Glycogen Values of Livers of Alloxan-Diabetic Rats, with and without Insulin Injections, as Determined by Reducing Value of the Hydrolyzed Glycogen and by Iodine Adsorption of the Intact Glycogen Molecule

Rat	Treatment	Glycogen as glucose p. cent of wet weight	
		By reducing value	By iodine adsorption
4	None	5·55	5·32
5	None	3·65	4·36
6	None	2·36	2·80
7	Insulin treatments for 12 h[1]	11·0	19·7
8	Insulin treatments for 12 h[1]	12·4	21·8
9	Insulin treatments for 24 h[2]	13·5	16·8
10	Insulin treatments for 24 h[2]	13·1	17·2
11	Insulin treatments for 24 h[2]	12·5	25·9
12	Insulin treatments for 24 h[2]	8·91	12·7
13	Insulin treatments for 48 h[3]	8·19	13·9
14	Insulin treatments for 48 h[3]	8·87	8·07
15	Insulin treatments for 48 h[3]	4·21	5·96
16	Insulin treatments for 72 h[4]	3·91	3·85
17	Insulin treatments for 72 h[4]	5·42	8·99
18	Insulin treatments for 72 h[4]	3·76	4·71

[1] Each rat received an injection of protamine-zinc insulin, 40 units per kilo body weight, 12 h before sacrifice, and the same dose of regular insulin exactly 2 h before sacrifice.

[2] Each rat received an injection of protamine-zinc insulin, 40 units per kilo body weight, 24 h before sacrifice and the same dose of regular insulin exactly 2 h before sacrifice.

[3] Each rat received injections of protamine-zinc insulin, 40 units per kilo, gody weight, at 48 h and 24 h before sacrifice and the same dose of regular insulin 2 h before sacrifice.

[4] Each rat received injections of protamine-zinc insulin, 40 units per kilo body weight, at 72, 48, and 24 h before sacrifice and the same dose of regular insulin exactly 2 h before sacrifice.

Table II.–A Comparison of Glycogen Values of Livers of Normal Rats, First Fasted 18 h, Then Intubated with Glucose, as Determined by Reducing Value of the Hydrolyzed Glycogen and by Iodine Adsorption of the Intact Glycogen Molecule

Rat	Treatment*	Per cent glycogen as glucose (wet wt. basis)	
		By reducing value	By iodine adsorption
1	Sacrificed 3 h after intubation	1·24	1·33
2	Sacrificed 3 h after intubation	1·20	1·09
3	Sacrificed 6 h after intubation	3·21	4·58
4	Sacrificed 6 h after intubation	4·84	8·71
5	Sacrificed 9 h after intubation	8·43	13·2
6	Sacrificed 9 h after intubation	5·68	9·11
7	Sacrificed 12 h after intubation	6·27	10·7
8	Sacrificed 12 h after intubation	8·38	14·4
9	Sacrificed 18 h after intubation	10·6	27·3
10	Sacrificed 18 h after intubation	13·6	28·9
11	Sacrificed 24 h after intubation	12·2	22·7
12	Sacrificed 24 h after intubation	9·42	14·4

* Rats were first fasted 18 h, then stomach-tubed with 3 gm of glucose in a 50% solution, and allowed access to the high carbohydrate diet until sacrificed.

In order to represent the degree to which values obtained by the iodine method deviated from those obtained by the titration method, we have made use of a ratio, R, which is:

$$R = \frac{\text{per cent tissue glycogen by colorimetric method}}{\text{per cent tissue glycogen by titrimetric method}}$$

R values were calculated from the data of each liver given in Table 1, and the average R value for each group was plotted against the duration of insulin treatments in Figure 1. The average R value for the uninjected, diabetic livers is given at zero time, and is approximately 1.

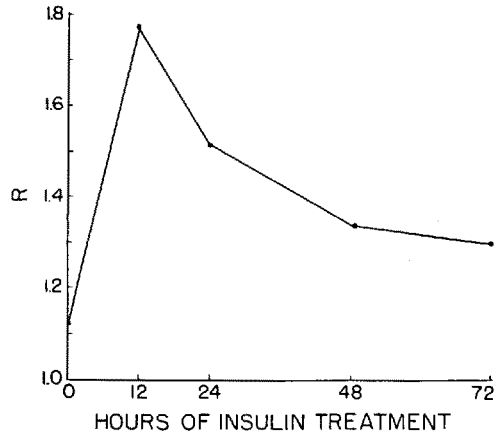


Fig. 1.–Change of ratio R (see text) with time in the case of diabetic rats injected with insulin. Rats received 40 units of protamine-zinc insulin per kilo body weight at 0 time and at each 24-hour period thereafter. In addition, each animal received 40 units of unmodified insulin just 2 h before sacrifice. Thus, rats were under the influence of insulin for 12 to 72 h. Each point represents the average of determinations on 2–4 animals.

The iodine adsorption characteristics of the liver glycogen of uninjected, alloxan-diabetic rats closely approximate those of the normal rats (the R value for liver glycogen of normal rats is taken as 1). It should be noted that the highest R value was observed in the early periods after the start of insulin treatments and that, with continued insulin administrations, the R values fell.

The shape of the curve shown in Figure 1 suggested that a high R value (i.e., a high iodine adsorption) might be characteristic of rapidly-formed glycogen. In order to test this hypothesis, experiments were carried out on rats that were first fasted for 18 h, to deplete their glycogen stores, and then intubated with glucose to induce rapid glycogenesis. Immediately after the intubation, these animals were fed *ad libitum* the high glucose diet, and were sacrified from 3 to 24 h after the intubation. The results are shown in Table II and Figure 2. Each point in Figure 2 is the average of the two R values obtained at a given time interval. The R value at the 3-h interval was essentially unity, but by the time 6 h had elapsed after the intubation of glucose, a marked rise in the value for R was observed, which attained a maximum at 18 h. These findings support the idea that liver glycogen that is rapidly deposited in response to (1) an insulin injection in the diabetic, or (2) glucose administration in a normal, fasted rat has a much greater iodine adsorption capacity than does liver glycogen of an animal in the steady state.

In order to rule out the possibility that the differences in iodine adsorption among the glycogen samples could

be due to the presence of iodine-adsorbing compounds other than glycogen, samples of glycogen prepared from diabetic rats and from insulintreated, diabetic rats were

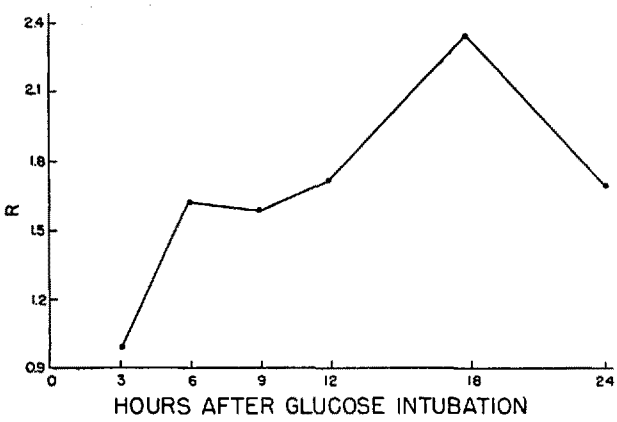


Fig. 2.–Change of ratio R (see text) with time in the case of norm a rats, first fasted for 18 h, then intubated with 3 gm of glucose at 0 time, and allowed access to a high carbohydrate diet thereafter. Points represent averages of determinations on pairs of animals sacrificed at the intervals indicated.

purified according to the method of SOMOGYI[1]. This procedure yields a product relatively free of salt, nitrogen, and phosphorus, and involves extraction with lipid solvents. The optical densities per milligram of glycogen were determined, and were referred to a sample of purified glycogen prepared from the liver of a rat in the steady state. At each time interval, the values for R were essentially the same as those shown in Figure 1.

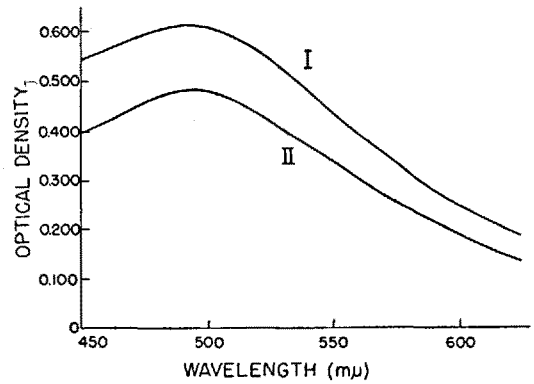
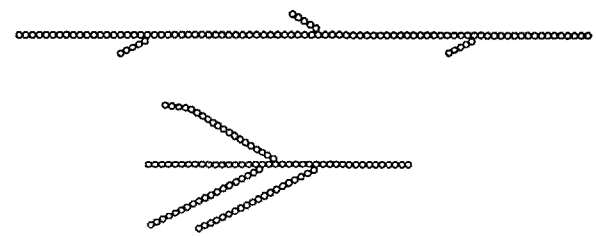


Fig. 3.–Absorption spectra of glycogen-iodine complex obtained with samples of purified glycogen. (i) Liver glycogen from a rat first fasted, then intubated with glucose, and sacrificed 6 h later. (ii) Liver glycogen from normal rat. Conditions of optical density measurements: glycogen, 0·1 mg per milliliter; KCl, 5 mg per milliliter; KI, 3 mg per milliliter; I_2, 0·5 mg per milliliter; T = 25°. Blank identical but without glycogen.

The optical densities referred to above were measured at a 500 mμ. The absorption spectra of two purified glycogen samples were also determined. One sample was obtained from a normal rat, the other from a rat that first had been fasted for 18 h, then intubated with 3 gm of glucose, and finally sacrificed 6 h after the glucose intubation. The results, shown in Figure 3, indicate that although the color intensity per unit weight of glycogen differed in the two glycogen samples, the values for R calculated from these spectra over the range of 450 to 700 mμ remained constant at 1·3. Thus, there is no apparent qualitative difference in the absorption spectra of the glycogen-iodine complexes of the two glycogen samples.

Discussion.–In the process of rapid glycogen deposition in the liver, a component appears with an iodine adsorption capacity relatively higher than that observed in glycogen found in the steady state. This higher iodine adsorption may result, in part, from the formation of a different species of polysaccharide or from a complexing moiety in relatively greater abundance than that present in steady-state glycogen. In experiments with diabetic rats which were first fasted for 24 h, then fed glucose and sacrificed 3 h later, LASZT[1] demonstrated, by fractional precipitation, the production of glycogen fractions which differ in their ability to complex iodine. Since he observed no difference in end-group analyses of these fractions, the essential basis for difference in iodine adsorption of various glycogen samples might lie in the distribution of glucose moieties within the molecule. This is illustrated in the following diagram by the two portions of glycogen molecules which, though having the same degree of branching, i.e., the same number of glucose moieties per end group, differ considerably in shape and distribution of chain length.



This difference is subject to detection by exhaustive phosphorylase degradation, and such a study, as well as a study correlating chain length with intensity of the resulting complex, is in progress.

Since the differences in the iodine adsorption of the various liver glycogen samples studied here are correlated with the age of the glycogen, it is not unreasonable to assume that the newlyformed glycogen contains many more molecules per unit weight than does a glycogen sample obtained from an animal in the steady state. The postulate that iodine adsorption in polysaccharides is a micellar surface phenomenon has received little support from experimental findings[2]. An explanation of our findings might lie in a less sterically-hindered approach of the iodine to the complexing site in the smaller molecule. The possible correlation of iodine adsorption ability with molecular weight is also the subject of investigation in this laboratory.

Data presented here and by other workers demonstrate that wide differences in iodine adsorption values for liver glycogen can be obtained from animals in a variety of experimental conditions or by the administration of various sugars[3]. However, the accumulated evidence indicates that the properties of the "abnormal glycogens" formed under such conditions is attributable to the rate at which glucose units are made available for glycogen synthesis.

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It is apparent from the findings presented here that colorimetric methods based on iodine adsorption cannot always be used for the quantitative determination of glycogen in liver. Certain difficulties encountered with the use of the iodine color method have already been pointed out by MORRIS[1]. He found that temperature, salt concentration, and source of "standard" glycogen are critical factors, and stressed the point that standard samples of glycogen should be isolated from the same tissue as that under investigation. But, as shown in the present study, the colorimetric method is further limited by the fact that the physical characteristics of the glycogen responsible for the color intensity of the iodine-glycogen complex are extremely sensitive to the physiological state of the animal. Thus, valid measurements of tissue glycogen cannot be made by the iodine method when a rapid deposition of the polysaccharide has taken place.

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Zusammenfassung

Es wird für die Bestimmung des Leberglykogens eine Jod-Adsorptionsmethode mit der üblichen Hydrolyse zu Glukose verglichen. Für normale und unbehandelte diabetische Ratten zeigen die Glykogenwerte mit beiden Methoden gute Übereinstimmung. Dagegen ist die Farbintensität je Gewichtseinheit des Jod-Glykogen-Komplexes bei insulinbehandelten diabetischen Ratten ebenso wie bei fastenden glukosegefütterten Ratten wesentlich grösser als bei normalen Tieren. Daraus lässt sich schliessen, dass frisch gebildetes Glykogen eine erhöhte Jod-Adsorptionsfähigkeit besitzt. Qualitativ erfährt die Farbe des Jod-Glykogen-Komplexes unter den verschiedenen Bedingungen keine Veränderung.

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STUDIORUM PROGRESSUS

Die Dehnbarkeit des Skelett- und Herzmuskels der Schildkröte während der Latenzzeit

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Die ersten Elastizitätsuntersuchungen am Muskel während der sogenannten Latenzzeit (das heisst der Zeit, die vom Augenblick des Reizes bis zum ersten eben nachweisbaren Anstieg der Spannung über die Ausgangsspannung verstreicht) sind von HILL[2] durchgeführt worden. Wenn der Muskel schnellen Dehnungen um etwa 10% seiner Länge unterzogen wird, dann steigt noch während der Latenzzeit die Spannung des gereizten Muskels stärker an als die des ruhenden, nicht gereizten Muskels. Aus diesem Befund schliesst HILL, dass das kontraktile Material infolge des Reizes weniger dehnbar werde, und zwar schon zu einem Zeitpunkt, in dem die Verkürzung der kontraktilen Elemente nach aussen noch nicht manifest werde. BUCHTHAL und ROSENFALCK[3] haben an Einzelfasern in isotonischer Anordnung durch periodische Änderung der Last gleichfalls die Elastizität während der Latenzzeit untersucht und wie HILL eine Abnahme der Dehnbarkeit vor Beginn der Kontraktion gefunden. Auch die Messungen der Torsionselastizität durch STEN KNUDSEN[1] haben dasselbe Resultat ergeben, obwohl natürlich ein Rückschluss aus diesen Befunden auf das Verhalten der Längselastizität nicht ohne weiteres möglich erscheint.

Nach unseren eigenen experimentellen Erfahrungen kommt es bei den Untersuchungen der Dehnbarkeit auf den mechanischen Ausgangszustand an, in dem sich der Muskel vor dem Versuch befindet. In der Arbeit von BUCHTHAL und ROSENFALCK[2] fehlen darüber nähere Angaben. Die Befunde von HILL sind an Muskeln erhoben worden, deren Ausgangslänge wesentlich kleiner als ihre Ausgangslänge *in situ* ist. Unter diesen Bedingungen rufen aufeinanderfolgende Dehnungen auch am ruhenden Muskel verschieden steile Spannungsanstiege hervor. Im nicht vorgedehnten Zustand hat der Muskel plastische Eigenschaften; seine Strukturen werden daher bei starken Dehnungen nicht nur elastisch beansprucht, sondern auch in ihrem Gefüge und in ihrer Anordnung geändert (REICHEL[3]). Solche plastischen Nebeneffekte erschweren eine eindeutige Aussage über das elastische Verhalten des Muskels erheblich. Bei der grundsätzlichen Bedeutung der Hillschen Befunde (s. WEBER und PORTZEHL[4]) halten wir es für notwendig, die Elastizitätsuntersuchungen während der Latenzzeit mit einer Methode zu wiederholen, die plastische Nebeneffekte ausschliesst.

Methodik. Wir haben uns einer früher beschriebenen Methode (PIEPER, REICHEL und WETTERER[5]) bedient, mit der dem Muskel nach dem zum ersten Mal von BUCHTHAL und KAISER[6] angegebenen Prinzip sinusförmige Längenänderungen hoher Frequenz aufgezwungen werden. Die resultierenden Spannungsamplituden sind dann ein Mass für die Steifheit des Muskels oder für den reziproken Wert seiner Dehnbarkeit. Die benutzten Längenänderungen sind relativ klein (1–4% der sogenannten «Standardlänge», die der Muskel *in situ* einnimmt, s. HILL[7]). Als Ausgangsspannung wird in den meisten Fällen eine Spannung gewählt, die weniger als 1% der isometrischen Gesamtspannung im Gipfel der Einzelzuckung beträgt. Der Spannungsbereich, in dem die durch die Längenänderungen hervorgerufenen Spannungsamplituden sich bewegen, ist daher sehr klein; das Minimum der Spannungsamplituden liegt gewöhnlich bei Null. Dadurch wird die Möglichkeit ausgeschlossen, dass nicht nur die kontraktilen Ketten, sondern auch etwaige parallel zu ihnen liegende Strukturen elastisch mitbeansprucht werden (über die Frage des sogenannten parallel elastischen Elements siehe im übrigen REICHEL[8]). Die Wahl einer niedrigen Ausgangsspannung bei kleinen Längenänderungen bietet ausserdem den Vorteil, dass die plastischen Nebeneffekte unbedeutend sind. Einzelne Versuche sind auch bei höheren Ausgangsspannungen durchgeführt worden. Soweit in diesen Versuchen plastische Effekte vorhanden sind, werden sie einige Zeit nach Ein-

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