Studies in Histochemistry. LXVIII. Determination of Glycogen in Microgram Samples of Tissue, Quantitative Histologic Distribution in the Rat Adrenal, and Influence of ACTH*

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Optimal conditions for determination of glycogen in microgram samples of tissue were established for the analysis, which involved removal of preformed glucose and its phosphate esters by methanol extraction, extraction of glycogen, acid hydrolysis, and measurement of the glucose by a colorimetric glucose oxidase assay. The reliability of the procedure was confirmed by recovery experiments in which glycogen was added to microtome sections of rat liver. The presence of ascorbic acid, epinephrine, and norepinephrine were shown to have no influence on the analysis of rat adrenal. Glycogen was found to occur in highest concentration in capsule, fasciculata, and medulla of the rat adrenal. Administration of ACTH produced significant decreases in fasciculata and glomerulosa, slight decreases in reticularis and medulla, but marked increases in the capsular region. The net loss of adrenal glycogen following ACTH stimulation is interpreted as further support for some aspects of the Haynes-Berthet hypothesis of corticosteroid genesis. This involves generation of reduced triphosphopyridine nucleotide (TPNH), for steroid hydroxylations and other reactions, through enzymatic steps in which glucose-1-phosphate (G-1-P) is formed by phosphorylase action on glycogen and converted to G-6-P, which is oxidized with reduction of TPN.

Previous work from this laboratory dealing with the quantitative histologic distributions of glucose-6-phosphate (G-6-P) and 6-phosphogluconate (6-PG) dehydrogenases (Greenberg and Glick, 1960a) and of pyridine nucleotides (Greenberg and Glick, 1960b) in the rat adrenal, with and without the influence of ACTH *in vivo*, offered support to aspects of the Haynes-Berthet (1957) hypothesis for the mechanism of ACTH action on the adrenal. For further elucidation, the present study was extended to the quantitative histologic distribution of glycogen in the rat adrenal and the effect, *in vivo*, of ACTH. A micro-modification of the determination of glycogen was developed for analysis of microtome sections of tissue by the glucose oxidase assay of the glucose liberated on acid hydrolysis of glycogen.

Experimental Procedures

Preliminary extraction of the tissue with cold 80% methanol was employed to remove glucose, which would interfere in the determination of glycogen (Kemp and van Heijningen, 1954), and it was found that G-1-P would also be removed by this treatment. A mixture (4:1) of 5% trichloroacetic acid and 2.3 N HCl proved adequate both for extraction of glycogen from the tissue and for the subsequent hydrolysis to glucose.

In a check of the time required to complete the acid extraction of the glycogen in a boiling water bath from microtome sections of rat adrenal tissue, it was observed that the quantity extracted increased to maximum at 11 minutes, and continued extraction to 20 minutes had no further influence. Accordingly, an extraction time of 12 minutes was adopted as standard procedure.

In another control experiment, a check of the time required to complete the acid hydrolysis in sealed tubes in a boiling water bath revealed that maximum hydrolysis was obtained by 2 hours, with no further change to 3 hours.

Because of its great specificity, the glucose oxidase method for determination of glucose was employed. The procedure of Huggett and Nixon (1957) was adapted to measurement of millimicrogram quantities of glucose, and, after completion of the enzyme reaction, 2 N HCl was added to overcome instability of the final color (Guidotti *et al.*, 1961). Every step in the procedure was checked to insure optimal analytical conditions for the particular samples used.

Extraction of adrenal or liver tissue with trichloroacetic acid–HCl, followed by hydrolysis in a boiling water bath and measurement of glucose, gave the same analytical result as that obtained by preceding this treatment with digestion of the tissue with 30% NaOH in a boiling water bath and precipitation of the glycogen with ethanol. This is illustrated by the following experiment:

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TABLE I

NONINTERFERENCE OF ASCORBIC ACID, EPINEPHRINE, AND NOREPINEPHRINE IN THE GLYCOGEN ANALYSIS (ABSORBANCY VALUES, GLUCOSE ANALYSIS)

Glycogen (25 μg/ml)	Glycogen (25 μg/ml) plus:					
	Ascorbic Acid (μmole/ml)		Epinephrine (μmole/ml)		Norepinephrine (μmole/ml)	
	3	15	3	15	3	15
0.240	0.240	0.235	0.250	0.245	0.245	0.245
0.245	0.245	0.235	0.250	0.250	0.245	0.235
0.235	0.250	0.240	0.245	0.245	0.240	0.245

Each of eighteen sections of rat liver tissue was extracted for several minutes with 50 μl of cold 80% methanol. Each of nine of the sections was digested with 15 μl of 30% NaOH for 1 hour in a boiling water bath. This was followed by cooling, addition of 75 μl of 95% ethanol, standing for 2 hours at 4°, and centrifugation at 4000 × *g* for 10 minutes, after which the supernatant fluid was discarded. The glycogen precipitate was washed twice with 50-μl portions of cold 95% ethanol and then with ethanol-ether (1:1) before being centrifuged again at 4000 × *g* for 10 minutes. The precipitate was dried over silica gel *in vacuo* and, along with the other nine samples, was subjected to steps 6-15 in the Procedure, with the exception that 50, rather than 15, μl of trichloroacetic acid–HCl was used in step 6. The mean absorbancy found for the samples subjected to the alkali digestion and alcohol precipitation was 0.47 (S.D. 0.075), and the value for the other samples was 0.47 (S.D. 0.036).

Since relatively high concentrations of ascorbic acid, epinephrine, and norepinephrine occur in the adrenal, an experiment was included to test whether the presence of these substances would influence the analysis of glycogen. From Table I it may be seen that addition of the substances to glycogen prior to estimation of the latter, starting with step 8 in the Procedure, had no significant effect on the analysis.

Demonstration of quantitative recovery of glycogen added to microtome sections of liver tissue is given in Table II as a further test of the reliability of the procedure used.

Preparation of Samples.—Adrenal glands were obtained as described by Greenberg and Glick (1958) from male albino rats (Sprague-Dawley, Holtzman Co., Madison, Wis.) weighing 300–400 g, approximately 3 months old. The animals were singly housed in a constant climate room (78° ± 1° F, 30 to 50% relative humidity) with controlled illumination (lights on at 6:00 a.m., off at 6:00 p.m.), and instantaneous death was inflicted by a single hammer blow on the head at noon ±15 minutes. ACTH (25 mg/kg) in 1 ml of physiologic saline solution was injected subcutaneously into some of the animals 3 hours prior to sacrifice, and control animals received 1 ml of saline.

Fresh frozen microtome sections (2.3 mm diameter, 16 μ thick, 0.069 μl volume) were cut serially in a cryostat cabinet at −15° and handled as described previously (Grunbaum *et al.*, 1956). One section was brushed flat on an albuminized glass slide and stained with toluidine blue for histologic examination, the next two sections were placed in a reaction tube for extraction of glycogen, and the residue was used for protein-nitrogen analysis by the bromsulfalein method (Nayyar and Glick, 1954). This sequence was followed throughout the entire cortex and into the medulla of the adrenal.

TABLE II

RECOVERY OF GLYCOGEN ADDED TO SINGLE MICROTOME SECTIONS OF RAT LIVER (ABSORBANCY VALUES, GLUCOSE ANALYSIS)

Tissue Section (1.6 mm diameter, 16 μ thick)	Glycogen (0.25 μg)	Tissue + Glycogen	% Recovery
0.295	0.040	0.365	
0.310	0.040	0.340	
0.295	0.045	0.345	
0.315		0.350	
0.290		0.339	
Mean 0.301	Mean 0.042	Mean 0.348	101
	(0.5 μg)		
	0.080	0.350	
	0.080	0.400	
	0.075	0.400	
		0.400	
		0.375	
	Mean 0.077	Mean 0.385	102

Method for Determination of Glycogen

REAGENTS.—The following reagents were used: (1) 80% (v/v) methanol, (2) trichlorocetic acid–HCl mixture: 200 μl 2.3 N HCl added to 800 μl 5% trichloroacetic acid, (3) 2 N HCl, (4) 0.93 N NaOH, (5) glucose oxidase (Sigma) stock solution: 10 mg/ml 0.5 M phosphate buffer, *p*H 7.0, stored at −20°, (6) horseradish peroxidase (Sigma) stock solution: 1 mg/2 ml 0.5 M phosphate buffer, *p*H 7.0, stored at −20°, (7) *o*-dianisidine stock solution: 5 mg/ml 95% ethanol, stored at 4° and prepared fresh every week, (8) glucose oxidase reaction mixture: 100 μl glucose oxidase stock solution, 100 μl horseradish peroxidase stock solution, 10 μl *o*-dianisidine, and 790 μl 0.5 M phosphate

buffer, *p*H 7.0, mixture prepared immediately before use.

PROCEDURE.—(1) Place two fresh frozen microtome sections of tissue in the bottom of each reaction tube (27 mm long, 4 mm bore). A metal block with holes is used as a tube rack. (2) Remove block with tubes from cryostat and keep cold with solid carbon dioxide. (3) Add 50 μl 80% methanol to each tube; cap and mix. (4) Centrifuge at 4000 $\times$ *g* for 5 minutes and discard supernatant. (5) Dry residue in a vacuum desiccator over silica gel. (6) Add 15 μl trichloroacetic acid–HCl mixture to each tube; cap, mix, and place in boiling water bath for 12 minutes. (This treatment does not significantly affect the amount of protein measured by the method used.) (7) Mix again and centrifuge at 4000 $\times$ *g* for 5 minutes. (8) Transfer 10-μl aliquots of the clear supernatant fluids to glass tubes (50 mm long, 5 mm bore) and seal in a flame. (9) Hydrolyze in boiling water bath for 2 hours. (10) Cool tubes in running tap water and centrifuge for a few minutes at full speed in a clinical table-model centrifuge. (11) Cut off top of tubes with file or, preferably, by placing the glass against the edge of a motor-driven carborundum wheel. (12) Add 5 μl 0.93 N NaOH and mix. (13) Add 30 μl glucose oxidase reaction mixture to each tube; cap, mix, and digest at 37° for 45 minutes. (14) Add 15 μl 2 N HCl and mix. (15) Read absorbancy (Beckman spectrophotometer, model DU) at 400 mμ against a blank in which tissue sample is omitted. (16) Construct standard curve from measurements of 10-μl samples of glucose solution (10–50 μg/ml) subjected to the same treatment as unknowns. (17) Dry the residue in the tubes after step (7) in a vacuum desiccator over silica gel for determination of protein-nitrogen.

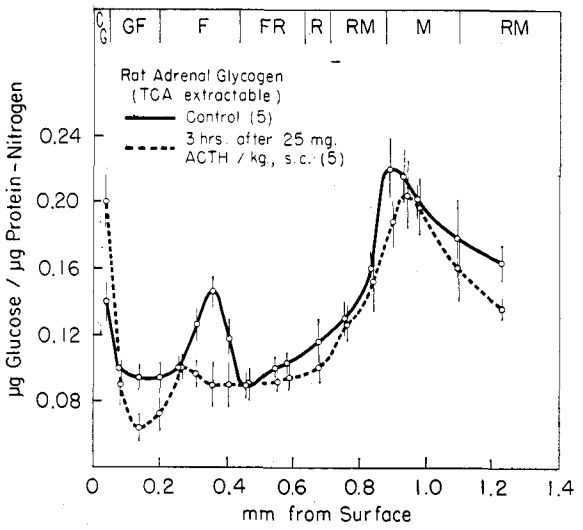

FIG. 1.—Quantitative histochemical distribution of glycogen in left adrenal glands of male albino rats. Two microtome sections (each 2.3 mm in diameter, 16 μ thick, 0.14 μl volume) were used for each analysis. The vertical bars represent one standard error of the means. The numbers in parentheses indicate the number of adrenals used to obtain the composite data. Regions marked G, F, R, and M denote, respectively, glomerulosa, fasciculata, reticularis, and medulla; mixed zones are designated by both letters.

RESULTS

The profile of the histologic distribution of glycogen in adrenals of untreated rats showed peaks in capsule, fasciculata, and medulla, with the highest concentration in the medulla (Fig. 1). Subcutaneous injection of ACTH caused significant reductions in glycogen concentrations in the fasciculata and glomerulosa, slight decrease in reticularis, marked elevation in capsule or the subcapsular region, and slight reduction in medulla.

DISCUSSION

Nicander (1957), using the periodic acid–Schiff (PAS) stain with a malt diastase control, reported localization of glycogen in the inner layers of the capsule, outer fasciculata, reticularis, and medulla, but none in the glomerulosa of the rat adrenal. Hunt and Hunt (1959), with the same procedure, found a similar distribution in adrenals of rats from 2 to 580 days old. In another similar staining investigation, Cohen (1961) observed a positive reaction for glycogen, primarily in fasciculata and reticularis, with weaker staining in the glomerulosa. Capsule and medulla were densely stained, but diastase resistant, and therefore considered devoid of glycogen. When rats were subjected to cold stress, the glycogen stain became negative in the fascicular and reticular zones, whereas capsule and medulla remained positive but were still diastase resistant. The variation between the latter and the earlier findings may be ascribed to differences in materials and technique. The quantitative data given in Figure 1 eliminate the ambiguity of the localizations based on the staining technique. The failure of Cohen (1961) to remove the glycogen present in capsule and medulla by treatment with diastase points to the need of caution in interpretation of this commonly employed procedure.

The loss of glycogen staining observed by Cohen (1961) in fasciculata and reticularis is in agreement with observations by Planel and Guilhem (1960), who found that administration of ACTH to rats caused almost complete disappearance of the stain in the adrenal cortex. The decrease in glycogen concentration following ACTH treatment is more precisely given by the data in Figure 1, and they support the results of Noble and Papageorge (1955), who reported a 40% decrease in glycogen concentration from whole adrenal glands of rats after intraperitoneal injection of ACTH (10 mg/kg of body weight).

The decrease in glycogen reported here, and the increase in G-6-P and 6-PG dehydrogenase

activities found earlier (Greenberg and Glick, 1960 a), after administration of ACTH, are consistent with the requirement of the hypothesis of Haynes and Berthet (1957) that TPNH generation be increased *via* the G-6-P dehydrogenase reaction, and that the source of G-6-P be G-1-P derived from glycogen. An increase in phosphorylase activity leading to production of G-1-P would also be expected, and this has been borne out by subsequent studies to be reported later.

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Studies on Glucose Phosphorylation in Rat Liver*

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Oxidation of glucose-C^{14} in fortified whole liver homogenates was strongly dependent on glucose concentration up to 0.1 M, whereas the oxidation of both glucose-6-P-C^{14} and fructose-C^{14} was maximal at low concentrations. With an assay procedure based on glucose-6-P formation as measured by TPN^+ reduction in the presence of an excess of purified glucose-6-P dehydrogenase, it was found that the glucokinase of rat liver has an apparent K_m of 0.01 to 0.04 M and V_{max} is reached at about 0.1 M glucose. TPN^+ reduction in this system in the presence of glucose and ATP was not appreciably inhibited by 0.06 M arsenate, whereas arsenate together with excess glucose-1-P yielded a reduction rate of less than one sixth that observed with glucose and ATP. Thus glucose-1-P was ruled out as the phosphorylation product of glucose in rat liver, and it is assumed that the direct phosphorylation product is glucose-6-P. These findings, taken in conjunction with previous data, indicate that glucose utilization in rat liver is regulated by a glucokinase whose activity is highly responsive to glucose concentration within physiologic ranges.

Despite the recognition of the central role played by the liver in carbohydrate metabolism, the mechanism of glucose utilization is obscure, owing in part to a lack of information concerning the properties of the phosphorylation system (Crane and Sols, 1955) and in part to the added complication that this organ not only utilizes but also produces glucose. A large and confusing body of literature exists which indicates that both uptake and release of glucose are influenced by hormonal and nutritional factors (Cahill *et al.*, 1959; Chernick *et al.*, 1951; Levine and Fritz, 1956; Jacobs *et al.*, 1958). The comprehensive studies of Cahill *et al.* (1958b) with rat liver slices demonstrate that glucose utilization is highly dependent on the glucose concentration of the medium and suggest that the glucokinase step may be rate-controlling. When these ob-

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† This work was carried out in part by David L. DiPietro as part of a doctoral thesis submitted to the Graduate Council of Temple University in partial fulfillment of the requirements for the Ph.D. degree. Present address: Department of Medicine, Harvard University Medical School, New England Deaconess Hospital, Boston 15, Massachusetts. A preliminary report of this work was presented at a meeting of the Biological Division of the American Chemical Society, September, 1960.