to a deflection of available energy necessary for transport[3] rather than to competition for a specific cellular constituent or "B substance" involved in the transfer of solutes across renal tubule cells.[8] It seems unlikely that the same B substance would be used in the transport of solutes in opposite directions within the cell, when solutes traveling in the same direction may have a different B substance, *e.g.*, glucose and glycine.[9] Assuming that the B substance of PAH is different from that of glucose, the cause of mutual interference must be sought in some portion of the transport mechanism common to each. As supporting evidence, Selkurt[3] has pointed out the fact that both Tm_G and $Tm_{diodrast}$ can be reduced by phlorizin.[2] In this regard, it seems more reasonable to suggest that the energy necessary for combination of either glucose or PAH with its B substance, that energy for the release from this substance at the terminus of transport, or that energy required for transport across the cell is not available in sufficient quantity to satisfy the simultaneous demands of PAH and glucose. In other words, this available energy may be the limiting factor in the capacity of the tubules when they are carrying these solutes in opposite directions simultaneously.

Conclusion. In the dog, the maximum renal tubular reabsorption of glucose (Tm_G) and the maximum tubular excretion of *p*-aminohippuric acid (Tm_{PAH}) are mutually depressed when determined simultaneously. The percentile depression of PAH by glucose is twice as great as the depression of glucose by PAH. This depression is attributed to competition for available energy rather than to competition for a common cellular constituent (B substance) involved in tubular reabsorption and excretion.

[8] Shannon, J. A., *Physiol. Rev.*, 1939, **19**, 63.

[9] Pitts, R. F., *Am. J. Physiol.*, 1943, **140**, 156.

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Improved Fixation for Histological Demonstration of Glycogen and Comparison with Chemical Determination in Liver.*

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Previous attempts to demonstrate a quantitative correlation between the amount of glycogen present in liver as determined by chemical procedures and as revealed by histological examination have been unsuccessful.[1,2] It is the purpose of the present paper to present data showing that under certain conditions such a correlation can be demonstrated. The increasing number of histochemical studies of tissues employing chemical technics on the one hand and histological technics on the other makes such a comparative study desirable.

This study was undertaken after it had been observed that glycogen can be preserved uniformly throughout histological blocks by placing pieces of organs in ice-cold fixative, instead of fixing blocks at room temperature as had been done heretofore.

Material and Methods. In order to prepare animals with variable concentrations of liver glycogen, a method originally suggested by Higgins, Berkson and Flock[3] was employed. For 5 days before sacrifice, 20 male

* This work was done in part under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

[1] Grafflin, A. L., Marble, A., and Smith, R. M., *Anat. Rec.*, 1941, **81**, 495.

[2] Eger, W., *Virchow's Arch.*, 1942, **309**, 607.

[3] Higgins, G. M., Berkson, G., and Flock, E., *Am. J. Physiol.*, 1932, **102**, 673; *ibid.*, 1933, **105**, 177.

Wistar rats, 3 months old and weighing on the average 307 g, were fed for a 2-hour period each day. Water was available at all times. The rats were killed by concussion, in groups of 4, at 6, 12, 18, 24, and 48 hours after the fifth and last meal. Pieces of the right and left lateral lobes of the liver were immediately dropped into separate bottles of Rossman's picro-alcohol-formalin fixative[4] chilled to 0°C. They were kept in this medium for 24 hours. Adjacent pieces of the 2 lobes, each weighing approximately 1 g, were immersed in weighed flasks containing 30% aqueous KOH at room temperature for the chemical determination of glycogen.

The flasks containing the samples of liver were reweighed and then immersed in boiling water for 30 minutes. Two aliquots of this alkaline digest were analyzed for glycogen by the Good, Kramer and Somogyi modification of Pflüger's method.[5] This method has been modified in the following respects: the whole procedure is carried out in 15 cc graduated centrifuge tubes; for the precipitation of the glycogen, 95% alcohol is added to give a final concentration of about 60%; after the mixture is heated to coagulate the precipitate, the tubes are chilled to 0°C in an ice bath before centrifugation; the supernatant fluid is then discarded, the precipitate dissolved in a minimum quantity of water, and the glycogen reprecipitated; finally, the hydrolysis of the glycogen is carried out by the method of Sjögren *et al.*[6]

The method of Folin and Wu[7] was used for the determination of glucose in the hydrolysate, and the amount of glycogen was calculated by means of the so-called Pflüger factor (0.927).[8] The difference in readings between the aliquots did not exceed 4% of their mean. The analytical results are recorded in Column 7 of Table I.

For the cytochemical preparations, the fixed blocks were embedded in 56-58°C paraffin in routine fashion; sections were carefully cut on a sliding microtome at 5 μ and stained for glycogen by the Bauer-Feulgen reagent following hydrolysis for one hour in 4% chromic acid.[9] After rinsing they were lightly counterstained with Harris' hematoxylin.

Estimations of the relative amounts of glycogen in the stained sections were initially tried by simple histological inspection of the intensity of staining. However, we have found a more satisfactory method for placing the preparations in order of glycogen content in the direct comparison of pairs of slides viewed simultaneously through a comparison ocular.

In order to obtain quantitative estimates of the glycogen content of the sections, 2 methods were employed. In the first the overall color of the section was compared with a standard solution of recolorized fuchsin by utilizing a comparison ocular attached to the eyepieces of the microscope and a Duboscq colorimeter.[10] This method proved only partly satisfactory since some difficulty was experienced in arriving at a reliable endpoint.

The second quantitative method, and the one by which the data reported in the paper were obtained, proved more reliable. The amount of light transmitted through the section was measured by means of an electronic light-meter (Photovolt photometer, model 512). The image of the section was projected at a magnification of 100× into the search unit of the photometer. A green filter (Wratten X) was employed to accentuate absorption by the fuchsin stain. The lighting was so arranged that the photometer scale recorded 100 when the light was passing through a clear slide and coverslip. The stained section was then interposed and the degree of light absorption recorded. Four readings, from different parts of the section, were taken for each preparation and averaged; from this figure was subtracted the average light-absorption by those sections exhibiting no glycogen detectable by the eye. The aver-

[4] Rossman, I., *Am. J. Anat.*, 1940, **66**, 277.

[5] Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 485.

[6] Sjögren, B., Nordenskjöld, T., Holmgren, H., and Möllerström, J., *Arch. ges. Physiol.*, 1938, **240**, 427.

[7] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

[8] Nerking, J., *Arch. ges. Physiol.*, 1901, **85**, 320.

[9] Bensley, C. M., *Stain Tech.*, 1939, **14**, 47.

[10] Dempsey, E. W., and Singer, M., *Endocrinology*, 1946, **38**, 270.

TABLE I.
Photometric and Chemical Determinations of and Histological Distribution of Glycogen in Livers of Rats Killed After a Known Feeding Time.

1	2	3	4	5	6	7	8	9
Hr since fed	Rat No	Optical density of sections l.l.	r.l.	avg	G glycogen/100 g tissue calculated	found	Diff.	Distribution in lobule
6	a	29.5	28.5	29	4.8	4.0	+0.8	even or p.c.
	b	30	37	33.5	5.5	5.1	+0.4	" " "
	c	25.5	29	27.3	4.5	4.9	—0.4	p.c.
	d	27	25	26	4.3	4.5	—0.2	"
					—	—		
					4.8	4.6		
12	a	31	28	29.5	4.8	5.0	—0.2	even or p.c.
	b	24	22.5	23.3	3.8	5.3	—1.5	" " "
	c	35	18	26.5	4.3	5.0	—0.7	" " "
	d	31	29	30	4.9	4.7	+0.2	p.c.
					—	—		
					4.5	5.0		
18	a	18	15	16.5	2.7	2.2	+0.5	c.c.
	b	17.5	16.5	17	2.8	3.1	—0.3	p.c.
	c	22	22	22	3.6	3.0	+0.6	"
	d	23	20.5	21.8	3.6	2.8	+0.8	"
					—	—		
					3.2	2.8		
24	a	0	3	1.5	0.2	0.5	—0.3	c.c.
	b	11	11	11	1.8	1.7	+0.1	"
	c	7	3	5	0.8	1.0	—0.2	"
	d	6	4.5	5.3	0.9	0.9	0.0	"
					—	—		
					0.9	1.0		
48	a	1	2	1.5	0.2	0.03	+0.2	invisible
	b	3	0	1.5	0.2	0.02	+0.2	"
	c	1	0	0.5	0.1	0.06	0.0	"
	d	0	0	0	0.0	0.08	—0.1	few cells scattered in c. zone
				—	—	—	—	
				$s_1 = 328.7$	0.1	0.05		
						$s_2 = 53.9$	s.d.±0.52	
				$s_2/s_1 = 0.164$				

l.l. = left lobe.
r.l. = right "
p.c. = peripheral concentration.
c.c. = central "

s_1 = total avg opt. densities.
s_2 = total found glycogen.

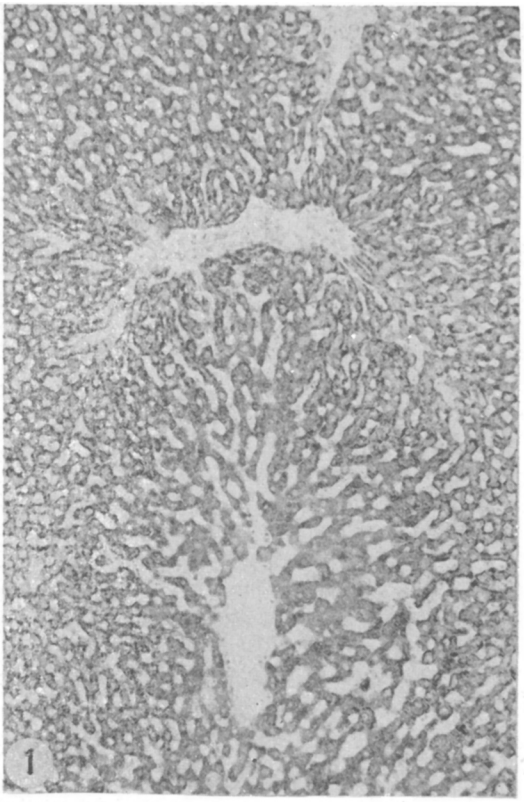
age differences among the 4 readings were ±2. The maximum variation between readings was 12 for liver No. 12d l.l., in which the extreme readings were 25 and 37. The corrected average readings of optical density are listed as Columns 3 and 4 of Table I. It will be noted that in the livers of rats 48 a, b and c, corrected photometer readings of 0.1-0.2 were made, even though there was no glycogen visible on microscopic examination. Such readings are, however, without significance and represent the limit of error of the method.

Results. The livers appeared to be uniformly and completely fixed. Unlike the usual histological preparations for glycogen[1] the sections possessed no rim of cells containing more glycogen than those in the center of the block. Moreover, as in frozen-dried preparations, glycogen was scattered in small particles throughout the cytoplasm rather than being concentrated against one membrane (Maximow and Bloom[11]). The quality of fixation is illustrated in the photomicrographs (Fig. 1-4).

Like the mouse,[12] the rat exhibits a zonation in the distribution of glycogen in the liver lobule, and Column 9 of Table I shows that the zonation undergoes a similar sequence of changes during starvation. Glycogen is great in amount and either evenly dis-

[11] Maximow, A. A., and Bloom, W., *A Textbook of Histology*, 1943, p. 15.

[12] Deane, H. W., *Anat. Rec.*, 1944, **88**, 39.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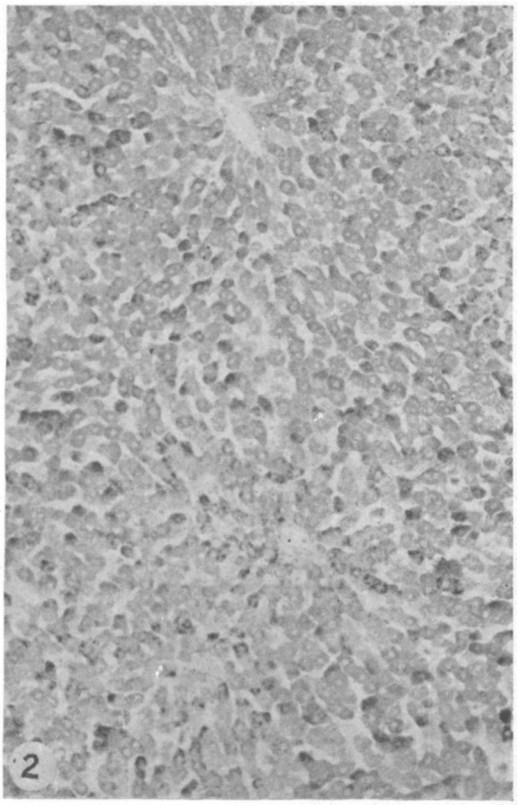
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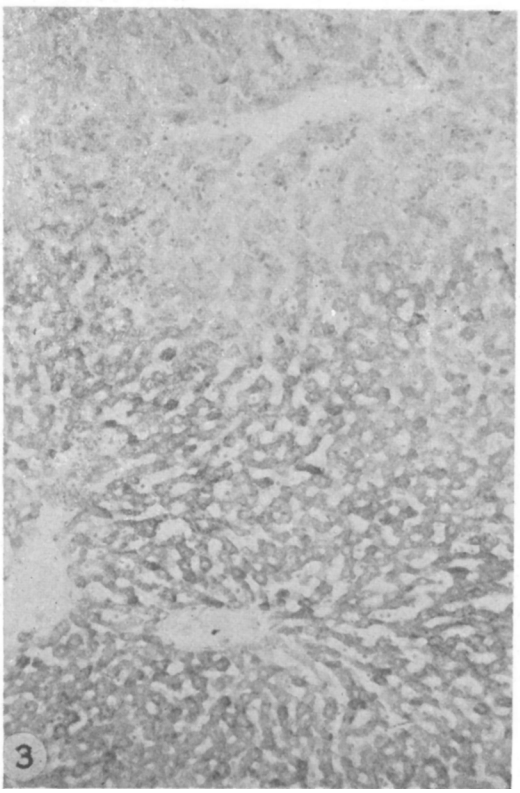
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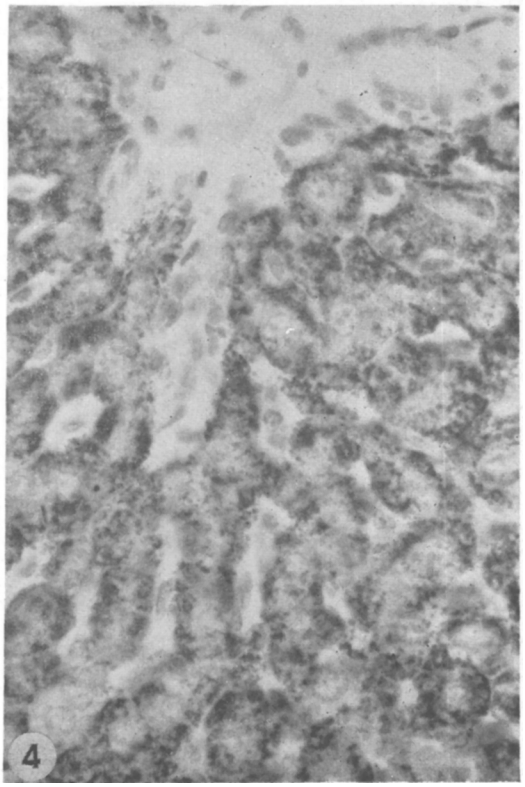
4

Photomicrographs of 5 μ sections of rat liver, fixed in ice-cold picro-alcohol-formalin and stained by the Bauer-Feulgen method for glycogen and lightly counterstained with Harris' hematoxylin. The pictures were taken with green and yellow filters combined (Wratten B and G) to accentuate the fuchsin color. They have all been printed on Kodabromide F1 paper to produce a comparable intensity of color.

FIG. 1. Rat 12d, left lobe of liver (glycogen content 4.7%, optical density 31). Marked concentration of glycogen, particularly in the peripheral zone of the lobule (above). The glycogen has not been "swept" across the cells by the method of fixation adopted here as is usual after fixation at room temperature. In the cells in the outer part of the lobule the greatest amount of glycogen lies adjacent to the sinusoids (See Fig. 4). ×120.

FIG. 2. Rat 18d, right lobe of liver (glycogen content 2.8%, optical density 23). Moderate amount of glycogen, about evenly distributed in the lobule but slightly more concentrated in the peripheral zone (above). The carbohydrate is evenly distributed in each cell although unevenly concentrated in different cells. ×120.

FIG. 3. Rat 24d, left lobe of liver (glycogen content 0.9%, optical density 6). Only slight amount of glycogen, occurring solely in the central part of the lobule (below). ×120.

FIG. 4. Rat 6a, right lobe of liver (glycogen content 4%, optical density 28.5). High power view of glycogen in cells near portal canal. It does not appear to have been displaced during fixation but to have been precipitated *in situ* (*cf.* Fig. 13 in Maximow and Bloom[11]). In peripheral cells glycogen is concentrated in the cytoplasm near the sinusoids. ×600.

tributed or peripherally concentrated in the lobule 6 and 12 hours after feeding (Fig. 1). It is reduced in amount and generally peripherally concentrated at 18 hours (Fig. 2), very low and centrally concentrated at 24 hours (Fig. 3), and virtually absent at 48 hours. Inspection of Table I shows that the shift from peripheral to central concentration occurs when the glycogen level is approximately 2%, and furthermore that the lowest amount of glycogen which is microscopically visible is 0.08%. This small amount of glycogen occurs in scattered cells only and is not detected by the photometric

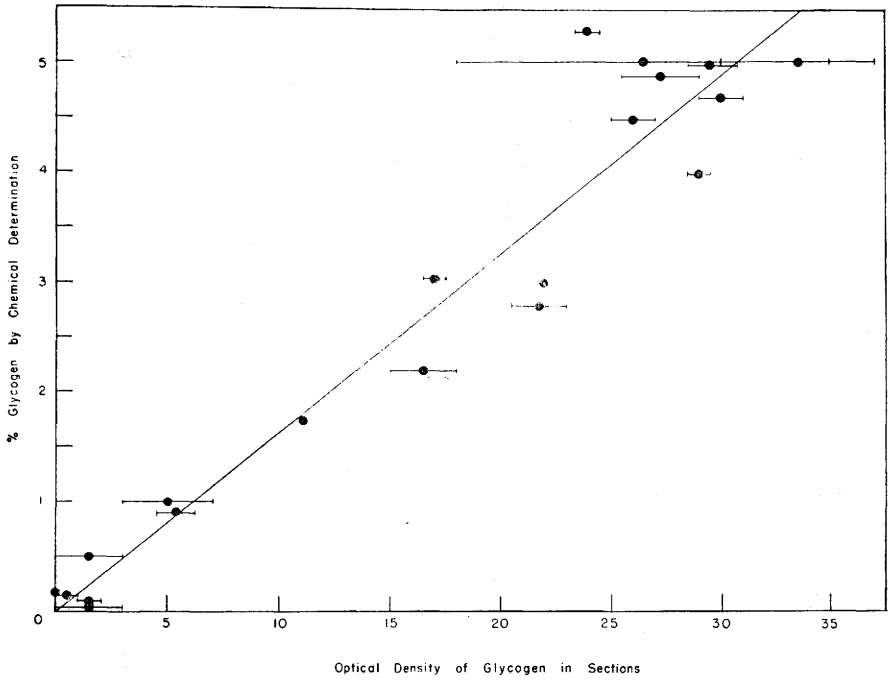

FIG. 5.

The black dots denote the chemically determined glycogen in a liver plotted against the optical density of the glycogen in the sections. The horizontal lines through the dots show the differences between the optical densities of the right and left lobes. The diagonal line is satisfied by the equation: % Chemically Determined Glycogen = 0.164 × Optical Density.

method. Interestingly enough, when there is a large amount of glycogen in the cells near the periphery of the liver lobule, it appears to be most concentrated in the cytoplasm adjacent to the sinusoids (Fig. 1 and 4).

The appraisal of the amount of glycogen in histological preparations is hampered by the unequal zonation of glycogen in the liver lobule. It was also found that lobules of the same liver lobe may possess different quantities of glycogen. This is true even when the sections are entirely uniform in thickness. Moreover, the 2 lobes occasionally present significantly different levels of glycogen (*e.g.*, No. 6b, 12c, and 24c). The possibility of such differences in distribution needs to be taken into consideration by both histologists and biochemists in drawing conclusions from small samples of liver.

The analytical data (Column 7) and the average corrected photometer readings (Column 5) of the livers of the individual rats are recorded in Table I by groups, according to the lapse of time after the last feeding. The average ratio of glycogen found/photometer reading = 0.164. Multiplying the individual photometer readings by this factor gives a calculated value of the glycogen in the histological sections of each rat liver (Column 6). The standard deviation of the differences between the found and calculated glycogen values is ± 0.52.

The data for chemically determined glycogen have also been plotted against the corrected photometer readings in Fig. 5. The black dots designate the average of the optical densities of the sections of a liver and the corresponding chemical glycogen determinations. The horizontal lines through the dots indicate the differences between photometer readings for the right and left lobes. It is seen that up to a glycogen concentration of about 5% a linear correlation exists between the 2 methods of determination.

It may be further pointed out that though no significant difference occurred in the liver glycogen between the 6- and 12-hour postfeeding periods, a marked progressive decrease occurred between the 12- to 18- and the 18- to 24-hour periods.

Summary. Glycogen of rat liver was well preserved throughout histological blocks and was not displaced in the cells when pieces of liver were fixed in ice-cold picro-alcohol-formalin. After fixation, liver sections were stained by the Bauer-Feulgen method and the optical density of each section was measured on a Photovolt electronic photometer. Chemical determinations were made of the glycogen contained in other pieces of the same livers. The livers of rats undergoing progressive starvation from 6 to 48 hours showed good correlation between the chemically and histologically determined glycogen concentrations.

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A Virus Causing Abnormal Milk in Cattle.

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Dairy cows near Princeton, N. J., have shown an unusually high incidence of bloody thickened milk during the spring, summer and fall of 1946. The majority of cases occurred in the latter part of May and in June and animals in all stages of lactation were affected. Many specimens of abnormal milk contained flakes composed of polynuclear and mononuclear cells. Affected animals frequently showed temperatures exceeding 40°C but otherwise appeared normal and continued to eat as usual. In most cases all visible signs disappeared after 2 to 4 days. These facts suggested an infectious nature and in-