THE EFFECT OF GLYCOGEN DEPOSITION ON LIVER PHOSPHORUS [1, 2]

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It has been a common concept that the deposition of glycogen within liver cells is accompanied by an accretion of water, potassium, and phosphate from outside the liver. The purpose of this paper is to show that glycogen deposition is indeed accompanied by the accretion of water and potassium, as originally demonstrated by Fenn (1), but that no phosphate enters the liver during glycogen deposition. We have found that the cells of livers low in glycogen contain more phosphate than can be completely ionized in the cell water if electroneutrality and osmotic equilibrium are to be maintained. As glycogen levels rise in the cells, accompanied by the accretion of water and potassium the amount of this excess phosphate diminishes in a fashion mathematically consistent with dilution. We therefore postulate that the anion equivalency and osmotic force required to maintain electroneutrality and osmotic equilibrium in the intracellular water that is deposited with glycogen are supplied by the conversion of acid-insoluble phosphate already present in the liver, to acid-soluble phosphate esters and orthophosphate.

METHODS

The data presented were obtained from the livers of 15 rabbits. Four groups of rabbits were included: normal rabbits, alloxan-diabetic rabbits, normal rabbits which had received cortisone (7.5 mg. for 16 to 18 days) and diabetic rabbits which had received cortisone.

Following intravenous Nembutal® anesthesia, the animals were exsanguinated, and their livers removed *in toto*. The livers were weighed and one sample placed immediately in 30 per cent KOH for glycogen determination. A second sample was placed in a weighing bottle for the determination of fat, water, nitrogen, and electrolytes.

Serum water was determined using a micropyknometer. Serum sodium and potassium were measured on an internal standard flame photometer. Chloride was determined by the method of Wilson and Ball (2). The Somogyi-Nelson method (3) was used for the determination of blood sugars. Inorganic serum phosphate was measured by the molybdenum-blue reaction as described by Fiske and Subbarow (4). A modification of Folin's method (5), using Nessler's reagent, was used in the determination of non-protein nitrogen.

The livers were analyzed for water by drying to constant weight at 100 to 102° C. Neutral fat was determined by extracting the powdered dry livers three times with a mixture of equal parts of ethyl and petroleum ether (6). Sodium, potassium, and chloride analyses were carried out on aliquots of a 0.75 N redistilled nitric acid extract of dry fat-free tissue (7). Sodium and potassium were determined by internal standard flame photometry and chloride by a micro-modification of the method of Wilson and Ball (2). For the determination of total phosphate, samples of dry fat-free liver powder were predigested with concentrated nitric acid, followed by perchloric acid until a clear, colorless solution was obtained. This solution was transferred quantitatively to a volumetric flask and aliquots used for the determination of total phosphate by the molybdenum-blue reaction. Total nitrogen was measured on an aliquot of tissue which was predigested with sulfuric acid and superoxol. The resulting clear, colorless solution was cooled, transferred quantitatively to a 25 ml. volumetric flask, and made to volume. Aliquots of this solution were further digested in a sulfuric-phosphoric mixture and the nitrogen determined by Nesslerization.

Calculations

Determination of the intracellular concentrations of phosphate, potassium, and sodium was made using the chloride space as a measure of extracellular water, as described by Hastings and Eichelberger (6). The application of this calculation assumes that the intracellular phase of the tissue contains no chloride, an assumption that is probably correct under physiological conditions, but may be incorrect under conditions of abnormal metabolism. The following equations present the method of calculation.

All analyses are calculated on fat-free tissue values.

$(Cl)_s$ = mEq. Chloride per liter serum.

$[Cl]_s$ = mEq. Chloride per kilogram serum water.

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[2] Published in abstract form in the proceedings of the 46th Annual Meeting of the American Society for Clinical Investigation, J. Clin. Invest., 1954, 33, 956.

$[Cl]_e$ = mEq. Chloride per kilogram extracellular water.
$(Cl)_K$ = mEq. Chloride per kilogram liver.
$(H_2O)_s$ = Grams water per liter serum.
$(H_2O)_K$ = Grams water per kilogram liver.
$(H_2O)_e$ = Grams extracellular water per kilogram liver.
$(H_2O)_i$ = Grams intracellular water per kilogram liver.
$(I)_K$ = mEq. ion per kilogram liver.
$(I)_e$ = mEq. ion per extracellular phase of a kilogram of liver.
$(I)_i$ = mEq. ion per intracellular phase of a kilogram of liver.
$[I]_i$ = mEq. ion per kilogram of intracellular water.
0.96 = Donnan factor.

(1) $\frac{(Cl)_s}{(H_2O)_s} = [Cl]_s$ (4) $(H_2O)_K - (H_2O)_e = (H_2O)_i$

(2) $\frac{[Cl]_s}{0.96} = [Cl]_e$ (5) $(I)_K - (I)_e = (I)_i$

(3) $\frac{(Cl)_K}{[Cl]_e} = (H_2O)_e$ (6) $\frac{(I)_i \times 1000}{(H_2O)_i} = I_i$

Definitions

The following definitions are used throughout this paper in discussing phosphate. They are based on Umbreit, Burris, and Stauffer's classification (8).

Total phosphate: All of the phosphate present in the tissue (the sum of acid-soluble and acid-insoluble phosphate).

Inorganic (ortho) phosphate: Phosphate that is present as the dissociation products of phosphoric acid. This phosphate is not in combination or equilibrium with any organic compound.

The dissociation constant for the over-all equilibrium of phosphoric acid at pH 6.9 is taken to be 1.3 (9).

Acid-soluble phosphate: Phosphate that is soluble in 10 per cent trichloracetic acid. This includes inorganic phosphate, phosphate esters such as the glucose phosphates which, in solution yield orthophosphate, and phosphate esters such as ATP and ADP, which are pyrophosphates and, on dissociation may not yield orthophosphate. Since the pKs of all of the orthophosphate esters are lower than that of phosphoric acid, and the pKs of ATP and ADP are similar (10), it follows that the dissociation constant of acid-soluble phosphate as a whole must be at least 1.3, and may be greater.

Acid-insoluble phosphate: Phosphate that is insoluble in 10 per cent trichloracetic acid, is bound to protein, and is presumably not ionized in cell water. This phosphate includes phospholipids and nucleoproteins.

RESULTS

Table I presents the basic data obtained. The livers of the animals receiving cortisone were markedly increased in size, both in normals and diabetics, and contained high concentrations of glycogen. As the glycogen concentrations in these livers increased, the concentration of total phosphate fell, while the concentrations of water and potassium remained relatively constant. Nitrogen concentrations fell as glycogen increased. Sodium and chloride showed no changes that paralleled the increase in glycogen content. Serum data showed normal values in all animals, with the exception of elevated blood sugars in diabetic animals.

Figure 1 illustrates the inverse relationship between total phosphate (as mM per 100 Gm. fat-free dry solids) and glycogen (Gm. dry glucose per 100 Gm. fat-free dry solids) found in these livers. The line was plotted from the formula of least squares and indicates that as the concentration of glycogen increases, the concentration of total phosphate decreases. In a liver with a concentration of 10 Gm. of glycogen per 100 Gm. FF dry solids there is a concentration of 45 mM of phosphate. As the concentration of glycogen increases, total phosphate falls, reaching at a level of 50 Gm. of glycogen, a value of 26.4 mM. The correlation coefficient for this line is 0.9564, with a z value of 1.90 ± 0.29, showing it to be highly significant.

DISCUSSION

From the data presented in Table I and Figure 1, it can be seen that, as glycogen is deposited in the liver, the concentration of total phosphate falls in a straight line, consistent with dilution.[3] Calculation of the intracellular concentration of phosphate in the liver with very low glycogen or high glycogen content, reveals that were all of the phosphate that is present in the tissue electrically and osmotically active in the intracellular water, neither electroneutrality within the cell or osmotic equality between intracellular and extracellular water would exist. Table II illustrates this.

This table presents the values for the calculated intracellular concentrations of potassium, sodium, and phosphate in a rabbit with a very low concentration of liver glycogen (No. 12), and a rabbit

[3] If a liver with 10 Gm. glycogen per 100 Gm. fat-free dry solids contains 45 mM of phosphate per 100 Gm. fat-free dry solids, then, if no phosphate is added to the liver as glycogen is deposited, the predicted phosphate concentration for a liver with 40 Gm. glycogen per 160 Gm. fat-free dry solids would be $49 \div 1.3$ or 34.6 mM of phosphate per 100 Gm. fat-free dry solids. The actual value found, taken from Figure 1 is 30.6 mM per 100 Gm. fat-free dry solids.

TABLE I

LIVER

Basic Data

Rabbit No.	Group	Liver Weight Gms.	% Body Weight	Neutral Fat Gms.	H_2O Protein Glycogen Gms./100 Gms. FF Dry Solids			Total Phosphate mM/100 Gms. F F Dry Solids	Potassium Chloride Sodium mEq/100 Gms. FF Dry Solids		
12	D	193	3.0	22	296	88.8	6.9	44.7	34.1	9.1	18.0
48	N	152	3.7	14	270	72.8	19.9	39.5	32.3	8.6	15.0
31	N	142	3.6	16	274	74.8	21.8	41.2	33.6	8.1	13.0
32	N	175	3.4	15	262	76.8	29.6	38.3	33.4	7.8	13.7
33	D	117	3.5	13	260	63.3	33.8	32.0	34.5	10.9	17.9
3	D	135	3.6	12	265	57.8	34.2	32.6	31.3	7.6	17.3
37	N	156	4.2	18	261	65.2	36.1	32.0	31.3	10.5	14.3
34	D + C	205	6.6	20	260	51.2	36.8	32.7	30.4	7.6	12.6
41	N + C	244	7.6	51	262	53.8	42.2	29.7	33.3	8.2	14.8
27	D + C	415	11.5	17	262	45.8	42.6	25.2	29.9	7.2	10.7
2	D + C	300	9.5	28	252	53.2	43.9	27.1	29.8	6.5	11.5
1	N + C	224	7.3	18	258	48.5	48.7	27.6	33.8	7.5	14.5
17	D + C	242	7.2	17	268	48.5	49.8	28.3	27.9	6.2	11.7
40	N + C	273	8.4	21	254	45.8	51.2	26.4	29.0	7.1	14.3
100	D + C	350	9.8	47	253	47.2	51.5	25.3	28.7	5.3	10.6

SERUM

Average of 15 Rabbits

H_2O Gms./L	Na	K	Cl	PO_4
	mEq/Liter			mgm/%
942	147	5.5	101	6.7
S.D. 5.77	2.89	0.16	5.4	0.89

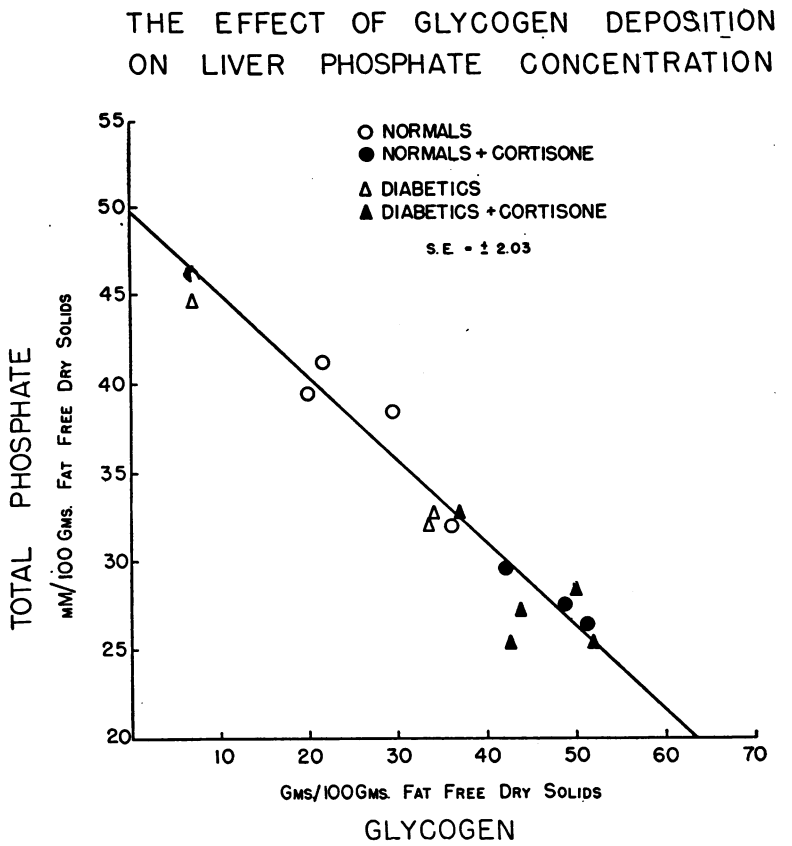


FIGURE 1

with a very high concentration of liver glycogen (No. 40), making the assumption that all liver phosphate is osmotically and electrically active. In the case of the rabbit with very low liver glycogen, there is a total of 397 milliosmols present in the intracellular fluid, as opposed to 320 in the extracellular fluid. (This does not take into account the additional milliosmols that must be present as magnesium, bicarbonate, sulfate, protein and organic acids.) Furthermore, the sum of the anions exceeds the sum of the cations. Assuming a dissociation constant of 1.3 for phosphate, there are 276 milliequivalents of phosphate present in association with 185 milliequivalents of sodium and potassium. Probably phosphate has a higher base-binding capacity than is indicated, because of the lower pK of carbohydrate esters. However, even if we assume a valence of one for phosphate, and assume that bicarbonate, protein sulfate and organic acids contribute no negative charges to the cell water, we still have 212 milliequivalents of phosphate present in the cell water—an excess of

TABLE II

Rabbit No. 12

Liver Glycogen = 6.9 Gm./100 Gm. FF Dry Solids

	mOsm.	*mEq.*
Potassium	161	161
Sodium	24	24
Phosphate	212	276 (f = 1.3)

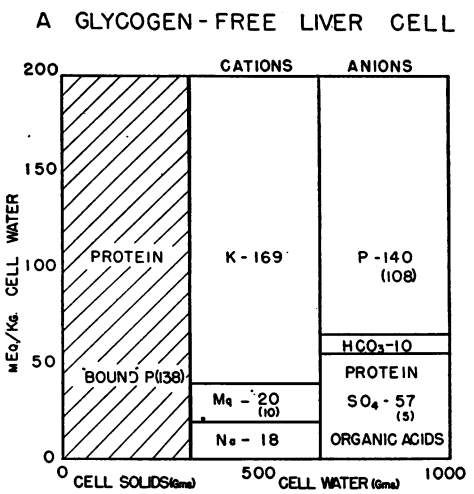
mOsm.	Extracellular Osmolarity	320
	Sum of Potassium + Sodium + Phosphate	397
mEq.	Sum of Sodium and Potassium	185
	Phosphate	276

Rabbit No. 40

Liver Glycogen = 51.2 Gm./100 Gm. FF Dry Solids

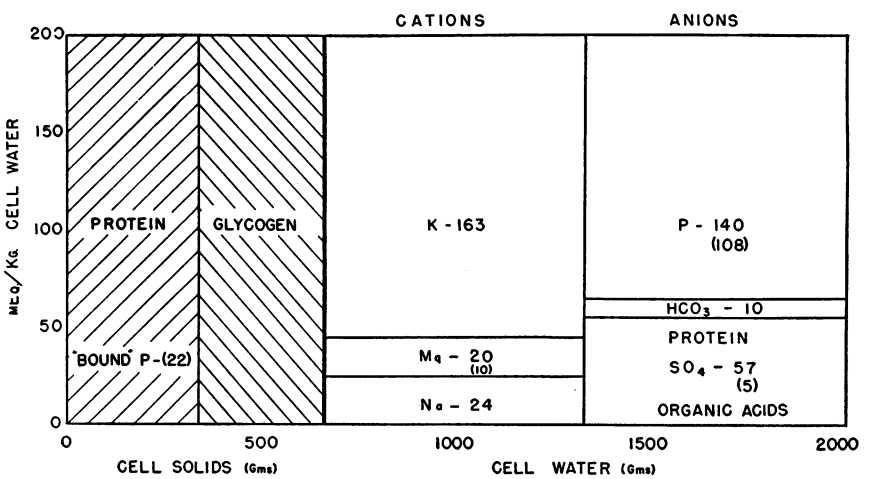
	mOsm.	*mEq.*
Potassium	146	146
Sodium	28	28
Phosphate	132	172 (f = 1.3)

mOsm.	Extracellular Osmolarity	320
	Sum of Potassium + Sodium + Phosphate	306
mEq.	Sum of Sodium and Potassium	174
	Phosphate	172



TOTAL ANIONS - 203 EXTRACELLULAR OSMOLARITY - 320
TOTAL CATIONS - 203 INTRACELLULAR OSMOLARITY - 320

CELL pH 6.93



TOTAL ANIONS - 203 EXTRACELLULAR OSMOLARITY - 320
TOTAL CATIONS - 203 INTRACELLULAR OSMOLARITY - 320

CELL pH 6.93

FIGURES 2 AND 3

1. The amount of *intracellular water* in a kilogram of cells is calculated as shown in the definitions (see Equation 5). The data used in Figures 2 and 3 is the average of the values for the 15 rabbits.

2. All of the *solids* (as calculated from wet weight − dry weight, corrected for fat content) are assumed to be intracellular. The data used in Figures 2 and 3 are the average value for the grams of solids associated with one kilogram of cells in the 15 rabbits.

3. Values for the concentration of *intracellular ions* were calculated from the equations presented.

4. *Potassium and sodium.* Figure 2: The average of the values for the two normal rabbits having the lowest liver glycogens (48 and 31). Figure 3: The average of the values for the two normal rabbits receiving cortisone having the highest liver glycogen (I and 40).

27 milliequivalents over the sum of sodium and potassium.

In the rabbit with high liver glycogen (No. 40), this large excess of phosphate is not seen—in fact, the osmolar sum of sodium, potassium and phosphate is only 306, compared to an extracellular osmolarity of 320, and the sum of the equivalent value of sodium and potassium is equal to the equivalent value of phosphate. However, if the other intracellular ions are added, there will again be a definite, small excess of milliosmols and anions. These data suggest that more phosphate is present in livers containing up to 50 grams of glycogen per 100 grams of dry fat-free solids than can be electrically or osmotically active in cell water. Furthermore, since there is no addition of phosphate to the liver during the deposition of glycogen, despite the addition of water and potassium, the osmotically and ionically active fraction of phosphate must increase with the deposition of glycogen at the expense of the bound phosphate. Fenn has shown (1) that the concentration of acid-soluble phosphate remains constant during the deposition of glycogen, while the concentration of acid-insoluble phosphate declines. Hence, it appears that the demand for more osmotically and electrically active phosphate ions during glycogen deposition is met by the conversion of acid-insoluble, or bound, phosphate, to acid-soluble phosphate esters and inorganic orthophosphates.

In contrast to the qualitative data presented in Table II, Figures 2 and 3 have been constructed to present a quantitative picture of the effect of glycogen deposition on the intracellular composition of liver. Certain assumptions necessary to the quantitative construction of these diagrams are presented below. The source of the data is indicated in the legend.

Assumptions

1) An intracellular pH of 6.9 was assumed throughout the calculations. This figure is based on data for muscle and may be inaccurate for liver, although the data of Cori and Cori on the pH limits for the activity of specific liver phosphatase (pH 6-7) suggest that the intracellular pH lies close to 7.0 (12).

2) Acid-soluble intracellular phosphate has been assumed to have an anion equivalence of 1.3 per mole. This is calculated from the dissociation curve of phosphoric acid (9) and is, at best, a rough approximation. We do know that most acid-soluble phosphate esters have pK's which are lower than orthophosphate (10) and, therefore, the anion equivalency of intracellular acid-soluble phosphate may actually be greater than 1.3. Without quantitating all of the acid-soluble phosphate compounds present in the cell, one cannot derive the exact base-binding capacity of intracellular phosphate. Furthermore, it is at present impossible to quantitate the base-binding capacities of cell protein. Since it is the sum of these two anions that provide almost all of the anions in the cell, it is inevitable that there will be some error in the specific number of acid equivalents assigned to each.

3) The total amount of magnesium found in livers is in close agreement between different groups of workers (13, 14). However, there is little evidence concerning the ionization of liver magnesium. What few data there are favor its being bound in part (15, 16). For this reason, we have assumed that only two-thirds of the total magnesium in liver (10 of 15 milliosmols per Kg. intracellular water) exists in a free form. We have also assumed that magnesium is added to liver cells during the deposition of glycogen. There is no experimental evidence yet on this subject.

4) Very little is known about the intracellular concentrations of protein, sulfate, and organic acids. However, it is generally conceded that minor amounts of these substances do occur within cells (17, 18), contributing a small number of milliosmols (about 5) and a greater number of

5. *Magnesium.* The average value found by a number of workers is 15 millimols per kilogram of intracellular water. A figure of 10 millimols, with an anion equivalency of 20, was used in the construction of these diagrams, with the assumption that some of the magnesium is bound to cell protein, and is not in solution in the intracellular water.

6. *Phosphate.* The values for phosphate were taken from Figure 1. The figure 49.8 mM of phosphate per 100 Gm. of fat-free dry solids, used in the construction of Figure 2, was taken from extrapolation of the line to zero. Twenty-four mM per 100 Gm. of fat-free dry solids was the figure used to calculate the intracellular concentration of phosphate in the liver where glycogen constitutes 50 per cent of the solids.

7. *Bicarbonate.* From the data of Wallace and Hastings (11).

8. *Protein, sulfate, and organic acids.* From the data of Hastings (17) and Gamble (18).

milliequivalents (about 50) to intracellular fluid. (The large discrepancy between the osmolar and anionic value is due to the polyvalency of the protein molecules.) Their molecular concentration in intracellular fluid is so low that large errors in estimation are of little importance in the total osmotic sum. Errors in the estimation of the anionic equivalence of protein may be present, as phosphate may have a greater anionic equivalence than 1.3, as has been discussed.

Figures 2 and 3 each represent a liver cell. For the sake of simplicity, the cell in Figure 2 has been assumed to weigh 1000 Gm. This is indicated on the abscissa. The concentrations of electrolytes present (per Kg. of intracellular water) are plotted along the ordinate and are also marked in the cells. Where the osmolar value of an electrolyte differs from its equivalent value, the osmolar value has been placed in parentheses beneath the equivalent value. The cell water is divided into two columns to illustrate the fact that there must be equal concentrations of anions and cations within it.

Figure 2 represents a glycogen-free liver cell, in which the cell solids are composed completely of protein. In order to maintain osmotic equilibrium with the extracellular fluid, the intracellular water of this cell must contain 320 milliosmols per liter. On the side of the cations, sodium and potassium contribute 187 milliosmols and magnesium 10, making a total of 197 milliosmols. On the side of the anions, bicarbonate accounts for 10 and protein, organic acids, and sulfate account for about 5, raising the total number of milliosmols present to 212. If all of the phosphate that is present in glycogen-free liver were osmotically active in the intracellular water, it would be present at a concentration of 246 milliosmols per kilogram, giving a total intracellular concentration of 458 milliosmols, as opposed to an extracellular osmolarity of 320. Furthermore, assuming bicarbonate to contribute 10 equivalents and protein, sulfate and organic acids 57, phosphate, with a dissociation constant of 1.3, would contribute 320 equivalents, making a total of 387 anions present in association with only 207 cations. A more feasible situation is the one which is represented in Figure 2 in which only 108 milliosmols of phosphate, yielding 140 milliequivalents, are present as acid-soluble phosphate in the intracellular water. This gives the intracellular water a total osmolarity of 320 and a total of 207 anions present with 207 cations. The remainder of the phosphate, some 138 millimols, must exist in the cell in the acid-insoluble fraction, in an osmotically and electrically inactive form, probably bound to protein.

Figure 3 represents a liver cell containing a high concentration of glycogen. Fifty per cent of the dry solids are glycogen. The remainder are protein. The deposition of this glycogen, accompanied by water, has caused the cell to increase 100 per cent in size. Assuming that the concentration of magnesium in the cell water remains constant, the sum of the cations has not changed. However, there has been a marked drop in the concentration of total phosphate. This drop is compatible with dilution of the phosphate present in the glycogen-free cell by the glycogen and water added, for it follows a straight line, as seen in Figure 1. Since the osmolarity of the extracellular fluid does not change in the animal with high liver glycogen (see Figure 1) the osmolarity of the intracellular fluid must also remain 320. The osmolar sum of the cations within the cell is again 197, and the anions bicarbonate, protein, organic acids and sulfate are presumed to contribute 15 more milliosmols, raising the osmolarity of the cell water to 212. If all of the phosphate that is present in a liver containing 50 grams of glycogen per 100 grams of dry solids were osmotically active in the intracellular water, there would be 130 milliosmols of phosphate, yielding a total osmolarity of 342, as opposed to the extracellular osmolarity of 320. However, if we again assume that 108 milliosmols of this phosphate are in solution in the intracellular water, osmotic equality exists between the extracellular and intracellular fluids and if this phosphate has a dissociation constant of 1.3, electroneutrality is achieved between cations and anions.

From these data, it appears that the liver cell containing 50 grams of glycogen per 100 grams dry fat-free solids has only 22 millimols of phosphate remaining in a bound form. These 22 millimols are probably associated with phospholipid and nucleic acids. This is less phosphate than is found in the phospholipids and nucleic acid of normal livers. One must therefore postulate that there is a marked decrease in the phospholipids

and/or nucleoproteins of liver cells during glycogen deposition. The present investigation does not enable us to distinguish whether this is due to glycogen deposition *per se* or whether cortisone administration affects the concentration of these compounds.

Since it appears evident from these experiments that there is no addition of phosphate to the liver during the deposition of glycogen, one is led to deduce that the fall in serum inorganic phosphate observed after the ingestion or infusion of glucose is associated entirely with the peripheral utilization of glucose. This hypothesis has been suggested by other workers, notably Bolliger and Hartman (19) in 1925, and Pollack, Millet, Essex, Mann, and Bollman (20) in 1934. More recently, several clinical observations in support of this concept have been made. In 1952, Groen and his coworkers (21) showed that a drop in serum inorganic phosphate was obtained in a patient with a necrotic liver, but was absent in a patient with pronounced muscular wasting. Observations by Van Bekkum and Querido further substantiate this theory (22). They studied a group of patients with progressive muscular dystrophy and observed that the drop in serum inorganic phosphate following glucose administration was significantly lower than in normal individuals.

SUMMARY

1. As glycogen concentration increases in the liver, the level of total phosphate declines. No extrahepatic phosphate is added to the liver during the deposition of glycogen up to 16 per cent of the liver (by wet weight).

2. The liver contains, at all levels of glycogen studied, more phosphate than can be completely ionized in the intracellular water if osmotic equality with the extracellular fluid and electroneutrality within the cell are to be maintained.

3. Since during glycogen deposition, total phosphate concentrations in liver decline while acid-soluble phosphate concentrations remain constant, there must be a conversion of acid-insoluble phosphate to acid-soluble phosphate during glycogen deposition.

4. These results support the theory that the fall in serum inorganic phosphate associated with the infusion of glucose is a measure of the peripheral utilization of glucose.

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