Metabolism of Hexose Phosphate Esters

1. METABOLISM IN NORMAL AND ALLOXAN-DIABETIC RABBITS

2. METABOLISM IN ISOLATED RAT DIAPHRAGM AND THE INFLUENCE OF INSULIN

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Shortly after the discovery of insulin, it was shown by a number of investigators (Wigglesworth, Woodrow, Smith & Winter, 1923; Harrop & Benedict, 1924; Bollinger & Hartman, 1925) that there was a decrease in the inorganic phosphorus content of the blood in normal and diabetic animals following the injection of insulin. These observations first led to the hypothesis that insulin was concerned with the phosphorylation of carbohydrates, and a number of investigators tried to correlate the decrease in the inorganic phosphorus of the blood with an increase in the hexose monophosphate of muscle. Cori & Cori (1932) and Soskin, Levine & Hechter (1941), however, considered that the increase in hexose monophosphate of muscle is probably a secondary effect of insulin due to an output of adrenaline. Nelson, Rapoport, Guest & Mirsky (1942) suggested that the decrease in inorganic phosphorus of the blood following insulin injections could be accounted for at least in part by the increase in the acid-soluble phosphorus fraction of the liver.

Kaplan & Greenberg (1943, 1944), working with radioactive phosphate, concluded from their results that insulin accelerates the transfer of phosphorus from the blood stream to the tissues, increases the hexose monophosphate content of muscle, and (in agreement with the results obtained by Nelson *et al.* 1942) increases the phosphorylation processes in the liver, as shown by the increased incorporation of the injected radioactive phosphorus into the acid-soluble phosphorus fraction and into the labile phosphate groups of adenosine triphosphate.

Sacks (1945), also using radioactive phosphorus, studied the effect of insulin on phosphate turnover in cat striated muscle. He reported an increased turnover rate of phosphocreatine, adenosine triphosphate and glucose 6-phosphate. Haugaard, Marsh & Stadie (1951) studied the changes in the phosphorylated intermediates in rat diaphragm muscle after incubation in a glucose-containing medium in the presence and absence of insulin. They

concluded from their results that insulin greatly increases the turnover rate of high-energy phosphate.

Price, Cori & Colowick (1945) reported that the hexokinase reaction in muscle and brain extracts was inhibited in rats by previously injecting them with anterior pituitary extracts or by adding anterior pituitary extracts to *in vitro* preparations, and that this inhibition was released by insulin. They also reported that the hexokinase from alloxan-diabetic rats is of the same order as that obtained in preparations from normal rats previously injected with anterior pituitary extracts, and that the activity is again restored to normal by insulin. These observations produced a great deal of speculation that the action of insulin is concerned with the phosphorylation of glucose to glucose 6-phosphate, i.e. the hexokinase reaction. However, a number of investigators (see Stadie, Haugaard & Hills, 1950, for references) were unable to confirm the work of Price *et al.* (1945), and up to the present there is no conclusive evidence for the action of insulin being directly involved in the phosphorylation of glucose.

The work reported in the present paper was undertaken to obtain some further evidence for or against this much disputed hypothesis for the site of action of insulin in carbohydrate metabolism. Two lines of approach were adopted: (1) the *in vivo* metabolism of glucose 1-phosphate and glucose 6-phosphate was compared with that of glucose in normal and alloxan-diabetic rabbits* and (2) the *in vitro* metabolism by the isolated rat diaphragm muscle of some of the phosphorylated hexoses was compared with that of glucose in the presence and absence of insulin.

* Some of these results have been previously published in a preliminary note (Beloff-Chain, Bovet, Chain & Pocchiari, 1951). After our preliminary publication Kuether (1951) reported results of similar but less detailed experiments carried out with glucose 1-phosphate on alloxan-diabetic rats. His findings and conclusions were very similar to our own.

Studies with the isolated diaphragm from alloxan-diabetic rats are now in progress and will be reported later.

METHODS AND MATERIAL

Preparations

Glucose 1-phosphate (G1-P). The dipotassium salt was prepared according to the method of McCready & Hassid (1944) using the cation-exchange resin Zeo-Karb 215 and the anion-exchange resin De-Acidite B, or the corresponding Amberlite resins.

Glucose 6-phosphate (G6-P). The barium salt was prepared according to the method of Seegmiller & Horecker (1951) from glucose and tetrakisphosphoric acid (Cherbuliez & Weniger, 1946).

Fructose 1:6-diphosphate (F1:6-P). The barium salt was prepared according to the method of Neuberg & Lustig (1942) from brewer's yeast.

Fructose 6-phosphate (F6-P). The barium salt was prepared from F1:6-P by hydrolysis in *N*-HBr for 5 days at 32° according to the method of Neuberg, Lustig & Rothenberg (1943).

Preparation of sodium salts. The hexose phosphates were used in all experiments as solutions of the sodium salts.

The sodium salt of G1-P was prepared by passing a solution of the K salt through a cation-exchange resin, and washing the resin with water until the pH of the effluent was approximately 4. The solution was then adjusted to pH 7 with a few drops of 30% NaOH, and concentrated *in vacuo* to the desired volume.

The sodium salts of G6-P, F1:6-P and F6-P were prepared by adding a slight excess of Na_2SO_4 to a solution of the barium salt (in dilute acid in the case of F1:6-P); the barium sulphate was centrifuged off and washed twice with water, and the supernatant plus the washings were adjusted to pH 7 and concentrated *in vacuo* to the required volume.

Analytical methods

Glucose was determined by the method of Miller & Van Slyke (1936).

All P determinations were made using the method of Berenblum & Chain (1938).

Glycogen was determined using the procedure of Good, Kramer & Somogyi (1933) as modified by Stadie, Haugaard & Marsh (1951).

G1-P was determined in the protein-free filtrates of blood and in the urine by measuring the inorganic P present before and after 10 min. hydrolysis in $\text{N-H}_2\text{SO}_4$ at 100°. In the *in vitro* experiments the G1-P was determined by measuring the glucose liberated after a similar hydrolysis.

G6-P was determined by subtracting the inorganic P from the total P obtained on ashing in perchloric acid.

In the *in vivo* experiments corrections were made for the initial values obtained in the blood prior to the injection of the substances.

Although the methods used for the determination of G1-P and G6-P are not strictly specific it was considered that under the conditions of these experiments they substantially measure the phosphate esters under investigation.

F1:6-P and F6-P were determined by measuring the fructose according to the method of Roe (1934).

The concentrations of the hexose phosphates are expressed throughout as mg. of glucose or fructose present.

Procedure for in vivo experiments

Male rabbits, weighing approximately 2 kg., were used. To produce alloxan diabetes, a single injection (200 mg./kg. in 5% solution) of alloxan was given intravenously into the ear vein. During the hypoglycaemic period following this injection the animals were fed a high carbohydrate diet and given concentrated glucose solutions to drink. Blood glucose was determined on the third or fourth day after treatment and subsequently for a minimum period of 4 days prior to an experiment. Only animals with a blood-glucose level exceeding 250 mg./100 ml. blood for a minimum period of 4 days were considered as diabetic. The animals were kept on a diet consisting only of green vegetables for 24 hr. before an experiment, or for a period of 48 hr. in some experiments with diabetic animals to obtain blood-glucose levels comparable with the normals. In the latter group the diabetic state of the animal was always confirmed by a glucose tolerance test.

The substance under investigation was injected intravenously into the ear vein, at the level of 1 g./kg. (expressed as glucose) as a 10% solution.

Experiments in which both ureters were ligated to prevent excretion of the injected phosphate esters in the urine were carried out as follows. The rabbits were lightly anaesthetized with ether and a median incision was made in the abdominal wall at the level of the bladder; the ureters were rapidly ligated and the wound closed. The operation was carried out about 2 hr. before the injection, and the animals killed immediately at the end of the experiment. The substances tested were injected in half the normal dose (0.5 g./kg., expressed as glucose). Control experiments, in which rabbits were operated on as described above but without injection of test substances, did not show appreciable alterations in the blood glucose during the 6 hr. period following the operation.

In all experiments blood samples were taken immediately before and 3, 15, 30, 60 and 120 min. after injection. About 2.5 ml. of blood were taken from the heart and transferred to a small test tube containing two drops of heparin. A 0.1 ml. sample was taken for glucose analysis, and 2 ml. of blood were added to 2 ml. of 10% trichloroacetic acid and centrifuged. The protein-free filtrate was analysed as described above.

The animals were kept in metabolism cages; in some experiments the urine was collected as excreted over a period of 24 hr. after injection, in other experiments the urine was collected by means of a catheter at regular intervals.

In connexion with the *in vivo* experiments, tests for phosphatase activity in the blood were carried out using the following procedure. Blood was withdrawn from the heart of a normal rabbit and a minimal amount of heparin added to prevent clotting. The blood was immediately mixed with solutions of G1-P or G6-P in the proportion of 2 ml. of blood to 1 ml. of a 5% solution of substrate (expressed as glucose concentration) previously warmed to 37°. Samples were taken for analysis of inorganic P immediately after the addition of blood and after 1 and 2 hr. incubation at 37°. The protein was precipitated with 10% trichloroacetic acid and the inorganic P of the supernatant was determined.

Procedure for in vitro experiments

Male rats weighing 120–180 g. were used. All animals were fasted for 24 hr. prior to the experiment. The isolated rat diaphragm technique, first described by Gemmill (1940, 1941), was adopted.

The rats were killed by decapitation. The two hemidiaphragms (weighing approximately 100 mg. each) were rapidly removed and weighed on a torsion balance to the nearest mg. Each hemidiaphragm was then transferred to 3 ml. of medium contained in a Warburg vessel and previously chilled in ice. The medium was at pH 6.8 and identical with that used by Stadie & Zapp (1947), containing 0.04M- Na_2HPO_4 , 0.08M- NaCl and 0.005M- MgCl_2 . The sub-

a physiological saline solution and transferred to 2 ml. of 30% KOH.

Substrate utilization. Samples of the medium were taken before and after incubation and analysed by the methods described above.

Respiration. The O_2 consumption was measured by the usual Warburg manometric technique.

Insulin effect. An insulin solution (0.1 ml.), containing 3 units, was added to the medium giving a final concentration of 1 unit/ml. Insulin was added to one hemidiaphragm of each rat and the results compared with those for the other hemidiaphragm incubated without insulin.

2:4-Dinitrophenol effect. A solution of 2:4-dinitrophenol (DNP) (0.1 ml.) was added to the medium before incubation to give a final concentration of $4.83 \times 10^{-5}\text{M}$.

Fig. 1. Rate of disappearance of intravenously injected glucose and G1-P from the blood of normal and alloxan-diabetic rabbits with accompanying increase in blood inorganic phosphorus and glucose. 1 g./kg. injected. The alloxan-diabetic rabbits were maintained on a carbohydrate-free diet prior to experiments to reduce the blood glucose to normal. Experiments carried out on the same diabetic rabbit on two consecutive days. (a) Normal rabbit (glucose injection). (b) Alloxan-diabetic rabbit (glucose injection). (c) Normal rabbit (G1-P injection); O—O, glucose; ●—●, inorganic phosphorus; x—x, G1-P. (d) Alloxan-diabetic rabbit (G1-P injection); key as for (c).

strates under investigation were used in a final concentration of 2 or 0.2%.

The flasks were attached to manometers, oxygenated for 5 min. and then incubated for 90 min. at 37°. At the end of the incubation period the flasks were removed from the manometers and immersed in ice.

Glycogen synthesis. Equal parts, approximately one-third of each half of the diaphragm, were taken together, weighed and immediately immersed in 2 ml. of 30% KOH for the determination of the initial glycogen content. The remaining two-thirds of each hemidiaphragm were incubated as described above. After incubation the diaphragms were removed, blotted on filter paper, rapidly washed in

RESULTS

I. IN VIVO EXPERIMENTS

The metabolism of G1-P and G6-P has been studied and compared with that of glucose, in normal and alloxan-diabetic rabbits. Experiments with G1-P were carried out on five normal and seven diabetic rabbits, and with G6-P on nine normal and seven diabetic rabbits. Similar results were obtained for all the animals, and typical illustrative examples are given in Figs. 1 and 2.

It was found that both G 1-P and G 6-P, injected intravenously, rapidly disappeared from the blood of both normal and alloxan-diabetic rabbits. In normal rabbits the rate of disappearance from the blood, i.e. the tolerance curve, was similar to that of glucose. In the alloxan-diabetic rabbits the glucose

organic phosphorus and glucose levels reached a maximum in most experiments in 30–60 min. In the 24 hr. period following the injections a very considerable increase in the excretion of inorganic phosphorus in the urine of all animals was observed (Fig. 4).

Fig. 2. Rate of disappearance of intravenously injected G 6-P from the blood of normal and alloxan-diabetic rabbits with accompanying increase in blood inorganic phosphorus and glucose. 1 g./kg. injected. (a) Normal rabbit. (b) Alloxan-diabetic rabbit. ○---○, Glucose; ●—●, inorganic phosphorus; ×—×, glucose 6-phosphate.

level of the blood remained high for a considerable time after injection in striking contrast to G 1-P and G 6-P which disappeared at a normal rate. In three diabetic rabbits, glucose and G 1-P were injected simultaneously; the results of one of these experiments is shown in Fig. 3.

Fig. 3. Rate of disappearance from the blood of glucose and G 1-P injected simultaneously into an alloxan-diabetic rabbit. 1 g./kg. of each substance injected intravenously. ○---○, Glucose; ×—×, G 1-P.

Following the injection of G 1-P and G 6-P the blood glucose increased from 30 to 100 mg./100 ml. in normal rabbits, and, as was to be expected, more markedly in diabetic rabbits. The inorganic phosphorus of the blood also increased from 4 to 12 mg./100 ml. (Table 1). The results show that there was a marked variation in the increase in blood glucose in different animals. Both the in-

As the results obtained in the above experiments could be explained by a breakdown of the phosphate esters in the blood, *in vitro* experiments were carried out with blood from two rabbits to ascertain whether or not this breakdown occurs. The results given in Table 2 show that no significant amount of either

Table 1. Maximum increase of inorganic phosphorus and glucose in the blood following intravenous injection of G 1-P and G 6-P in normal and alloxan-diabetic rabbits

(Injected 1 g./kg. Results expressed as mg./100 ml. of blood. Each line represents the results of an experiment on one rabbit treated with G 1-P and one with G 6-P.)

G 1-P injected		G 6-P injected	
Increase in inorganic P	Increase in glucose	Increase in inorganic P	Increase in glucose
Normal animals			
4.2	47	8.6	—
8.8	60	11.2	—
7.5	70	9.3	92
8.5	108	14.4	44
10.6	35	6.6	27
		5.0	45
		9.0	52
		12.2	55
		9.0	46
Diabetic animals			
11.6	38	10.3	93
8.5	270	9.2	94
12	90	9.6	195
12	106	7.8	140
11	105	6.8	103
7.6	54	8.0	202
11.6	170	10	145

Fig. 4. Excretion of inorganic phosphorus in the urine of normal and alloxan-diabetic rabbits, before and after injection of G1-P or G6-P. ▨, Alloxan-diabetic rabbits; □, normal rabbits.

Table 2. The phosphatase activity of normal rabbit blood with G 1-P and G 6-P as substrates

(The reaction mixtures contained 2 ml. whole blood and 1 ml. of a 5% solution of substrate (expressed as glucose).)

Rabbit	Substrate	Time of incubation at 37° (hr.)	Inorganic P liberated (μg./ml. of substrate)	Substrate hydrolysed (%)
1	G 1-P	1	0	0
		2	0	0
	G 6-P	1	8.4	—
		2	28.6	0.28
2	G 1-P	1	0	—
		2	15	0.17
	G 6-P	1	37.2	—
		2	103.2	1.20

Table 3. Percentage of intravenously injected G 1-P and G 6-P excreted in the urine of normal and alloxan-diabetic rabbits

(Injected 1 g./kg. Mean values given ± s.e.m. Number of rabbits and range given in parentheses. Urine collected for 24 hr. following injection.)

G 1-P excreted (%)		G 6-P excreted (%)	
Normal	Diabetic	Normal	Diabetic
31 ± 3.6	31 ± 3.1	44 ± 6.4	55 ± 4.7
(7)	(7)	(9)	(5)
(14-43)	(22-43)	(25-90)	(44-65)

G 1-P or G 6-P is hydrolysed when incubated for 2 hr. at 37° with whole blood.

The percentage of injected G 1-P and G 6-P excreted in the urine of normal and diabetic rabbits during a period of 24 hr. following injection is given in Table 3. These values showed that there was no significant difference between the percentage excreted in the normals and the diabetics, and also that taking the mean values there was no significant difference between the excretion of G 1-P and G 6-P although in a few rabbits a very high excretion of G 6-P was observed.

In order to prevent excretion some experiments were carried out on normal and diabetic rabbits in

which the ureters were ligated 2 hr. before the injection of G 1-P and G 6-P (0.5 g./kg.).

The results of these experiments are given in Table 4 and show that: (1) in most animals the phosphate esters had completely disappeared from the blood 1 hr. after the injection, (2) the increase in inorganic phosphorus of the blood in relation to the amount of phosphate ester injected was, as would be expected, higher than in animals in which a large amount of inorganic phosphorus was excreted in the urine, and (3) the increase in blood glucose was, as in experiments with unoperated animals, very variable but in most cases was of the same order as previously observed. The maximum increase in blood inorganic phosphorus and glucose occurred 1-2 hr. after the injection.

Toxicity tests

In carrying out the experiments reported above it was frequently observed that the animals presented signs of acute intoxication immediately

following the injection of G 1-P or G 6-P, which, if the animals survived, disappeared in 5-10 min. It was therefore decided to test the toxicity of these substances when injected intraperitoneally into mice, and to compare their toxicity with an equivalent amount of inorganic phosphate, injected into mice under the same conditions. The results are given in Table 5, and show that there was no significant difference between the toxicity of G 1-P and G 6-P and that their toxicity was only slightly less than that of an equivalent amount of inorganic phosphate. There was, however, a considerably longer delay before the onset of the toxic action of the phosphate esters than after the injection of sodium phosphate. The LD₅₀ value at normal temperature was approx. 6.9 mg. P/20 g. mouse for G 1-P, G 6-P and for inorganic phosphate.

It was found in later experiments with rabbits that the toxic manifestations which appeared immediately after injection in some of the first experiments could almost always be avoided by

Table 4. *Concentration of intravenously injected G 1-P and G 6-P in the blood of normal and alloxan-diabetic rabbits with ligated ureters, and the accompanying maximum increase in blood inorganic phosphorus and glucose*

(Results expressed as mg./100 ml. blood. 0.5 g./kg. injected.)

G1-P injected				G6-P injected			
Concentration of G1-P in blood following injection		Increase in inorganic P	Increase in glucose	Concentration of G6-P in blood following injection		Increase in inorganic P	Increase in glucose
3 min.	60 min.			3 min.	60 min.		
Normal animals							
260	73	16	157	162	0	11	127
240	22	19	107	—	—	—	—
Diabetic animals							
141	13	11	120	100	0	10.2	70
158	0	9.8	50	130	0	9.6	200
—	—	—	—	169	60	11.4	115
—	—	—	—	95	0	10.8	190
—	—	—	—	127	0	11.3	70
—	—	—	—	110	0	10.6	35

Table 5. *Toxicity of G 1-P, G 6-P and sodium phosphate in mice*

(Mice weighing approx. 20 g. were injected intraperitoneally with substances tested. Exps. 1 and 2 carried out at 37 and 18°, respectively.)

Exp.	Dose (mg./20 g. mouse) expressed as		G 6-P		G 1-P		Sodium phosphate	
	Inorganic P	Glucose	No. of deaths/total no.	Times of death (min.)	No. of deaths/total no.	Times of death (min.)	No. of deaths/total no.	Times of death (min.)
1	3.4	20	0/5	—	0/5	—	0/5	—
	4.3	25	0/5	—	0/5	—	0/5	—
	5.2	30	3/5	100-305	3/5	140-310	5/5	50-80
	6.9	40	5/5	125-150	5/5	80-105	—	—
	8.6	50	5/5	50-110	5/5	60-100	5/5	40-50
2	5.2	30	1/5	110	0/5	—	0/5	—
	8.6	50	4/5	205-265	3/5	80-110	5/5	40-90
	11.2	65	4/5	180-240	5/5	120-240	5/5	40-50
	13.8	80	5/5	70-240	5/5	240-255	—	—

Table 6. *The effect of glucose, G1-P and G6-P on insulin convulsions in mice*

(Experiments carried out at 37°. Insulin was injected intraperitoneally as indicated, and as soon as convulsions started the mice were injected intraperitoneally with glucose, G1-P or G6-P in the doses indicated, expressed as mg./20 g. mouse. The control groups were untreated. In experiments in which some of the animals survived, the arbitrary time of 180 min. was taken in calculating the mean values.)

		Glucose injections		G1-P injections		G6-P injections	
	Controls	25 mg.	50 mg.	25 mg.	50 mg.	25 mg.	50 mg.
	Exp. 1. 0.02 unit insulin/20 g. mouse						
No. of mice	20	10	5	10	5	10	5
Time between convulsions and death (min.). Mean $\pm$ s.e.m.	14.5	101.6	97	56.9	37.8	42.3	33.6
	± 2.5	± 12.7	± 7.0	± 15.0	± 4.0	± 6.2	± 3.9
No. of animals surviving	0	4	1	1	0	0	0
	Exp. 2. 0.10 unit insulin/20 g. mouse						
No. of mice	20	—	10	—	10	—	10
Time between convulsions and death (min.). Mean $\pm$ s.e.m.	13.5	—	99.9	—	36.9	—	29
	± 2.1	—	± 13.3	—	± 5.1	—	± 5.8
No. of animals surviving	0	—	2	—	0	—	0

injecting the solutions slowly (about 2 min. for 20 ml.).

Effect of glucose, G1-P and G6-P on insulin convulsions in mice

The curative effect of the esters on insulin convulsions in mice was compared with that of glucose. The technique used for these experiments and the results are given in Table 6. Although a 50 mg. dose of glucose/20 g. mouse was insufficient to assure complete protection, this was the maximum dose tested owing to the toxicity of the phosphate esters at higher concentrations; in fact a 50 mg. dose of G1-P or G6-P was less effective in prolonging life than a 25 mg. dose.

The findings of Marks & Morgan (1927) that a subcutaneous injection of hexose mono- or diphosphate did not relieve symptoms of insulin convulsions in rabbits or mice can be readily explained in their experiments with mice by the fact that the dose of the phosphate esters injected (100 mg., expressed as glucose, per mouse) is toxic.

Our results show that both G1-P and G6-P prolonged the lives of the mice after onset of insulin convulsions, but were less effective than glucose; the reason for this is not clear.

II. *IN VITRO* EXPERIMENTS WITH ISOLATED RAT DIAPHRAGM MUSCLE

The metabolism of G1-P

The metabolism of G1-P by normal rat diaphragm has been compared with that of glucose as regards the uptake of substrate, glycogen formation and effect on respiration. The influence of insulin on the utilization of glucose and G1-P has also been compared.

Uptake of substrate. The results are given in Table 7. It appeared that the uptake of G1-P was

higher than that of glucose in the absence of insulin, but that insulin caused a highly significant increase in the uptake of glucose (as first observed by Gemmill & Hamman, 1941, and later confirmed by Stadie,

Table 7. *Effect of insulin on utilization of glucose and G1-P by rat diaphragm muscle*

(Results expressed as mg. of substrate disappearing from medium/g. wet wt. of diaphragm in 90 min. at 37°, in O₂ (mean values $\pm$ s.e.m.). Substrates, 0.2%; insulin, 1 unit/ml. of medium. A positive or negative insulin effect indicates an increase or decrease in substrate utilization, respectively.)

No. of rats	Substrate	Substrate uptake		Insulin effect
		No insulin	With insulin	
12	Glucose	4.40* ± 0.30	9.73 ± 0.69	+5.33 ± 0.33 $P < 0.01$
10	G1-P	7.08* ± 0.58	4.46 ± 0.57	-2.62 ± 0.33 $P < 0.01$

* In the absence of insulin there is a highly significant difference in the uptake of glucose and G1-P ($P < 0.01$).

1944; Krah1 & Cori, 1947; Perlmutter & Greep, 1948; Krah1 & Park, 1948; Villee & Hastings, 1949), and a significant decrease in the disappearance of G1-P.*

Glycogen synthesis. The results given in Table 8 show that the degree of glycogen synthesis both from glucose and G1-P was largely dependent on the initial concentration of glycogen present in the diaphragm, in accordance with the results of Hansen, Rutter & Samuels (1951). Therefore, the experiments were divided into groups depending on the initial glycogen content. When the initial con-

* Since the completion of this work the authors have observed that part of the G1-P is converted into F6-P in the medium.

Table 8. *Effect of insulin on glycogenesis from glucose and G1-P by rat diaphragm muscle*

(The diaphragms were incubated for 90 min. at 37° in 3 ml. of medium in O₂. Mean values are given $\pm$ S.E.M. Substrates, 2% glucose or G1-P. Insulin 1 unit/ml. of medium. Glycogen synthesis indicates the increase in glycogen over the initial value. A positive or negative insulin effect indicates an increase or decrease in glycogen synthesis respectively.)

No. of rats	Initial glycogen content of diaphragm		Substrate	Glycogen synthesis (mg./g. wet wt.) tissue		Insulin effect	P
	Limiting values	Mean values		No insulin	With insulin		
4	0.20-0.39	0.25	G1-P	0.88 $\pm$ 0.20	0.72 $\pm$ 0.11	-0.16 $\pm$ 0.12	0.85
5	0.40-0.59	0.49	Glucose	0.76 $\pm$ 0.14	1.57 $\pm$ 0.38	+0.81 $\pm$ 0.32	0.10
5	0.40-0.59	0.49	G1-P	0.72 $\pm$ 0.26	0.78 $\pm$ 0.25	+0.06 $\pm$ 0.04	0.85
8	0.60-0.79	0.69	Glucose	0.65 $\pm$ 0.15	1.09 $\pm$ 0.14	+0.44 $\pm$ 0.09	0.04
6	0.60-0.79	0.66	G1-P	0.70 $\pm$ 0.21	0.86 $\pm$ 0.26	+0.16 $\pm$ 0.01	0.60
6	0.80-0.99	0.88	Glucose	0.45 $\pm$ 0.16	0.60 $\pm$ 0.09	+0.15 $\pm$ 0.09	0.40
4	0.80-0.99	0.91	G1-P	0.47 $\pm$ 0.07	0.43 $\pm$ 0.08	-0.04 $\pm$ 0.06	0.70
6	> 1.00	1.25	Glucose	0.02 $\pm$ 0.18	0.58 $\pm$ 0.16	+0.56 $\pm$ 0.13	0.05
6	> 1.00	1.39	G1-P	0.12 $\pm$ 0.18	0.09 $\pm$ 0.19	-0.03 $\pm$ 0.06	0.90

Results are considered significant when $P=0.1$ or less.

centration exceeded 1 mg./g. wet weight of tissue there was usually little or no synthesis of glycogen and in some cases a glycogenolysis was observed.

In accordance with the findings of other authors (Gemmell, 1941; Stadie & Zapp, 1947; Corkill & Nelson, 1947; Perlmutter & Greep, 1948; Villee & Hastings, 1949) in most cases there was a significant increase in glycogen synthesis from glucose in the presence of insulin (Table 8). Only in the group with

those of the G1-P experiments. The results are given in Table 9, and show that the insulin effect is not altered by the increased ionic concentration of the medium. As only four rats were used for these experiments, no statistical analysis of the results was made.

Table 10. *Oxygen consumption by rat diaphragm muscle in the presence of glucose, G1-P and G6-P*

(Results expressed as μ l. O₂/100 mg. wet weight of diaphragm in 90 min. at 37° in O₂. Mean values given $\pm$ S.E.M. Substrates, 2% glucose, G1-P and G6-P. The hemidiaphragms from each rat were incubated in glucose and G1-P respectively, or glucose and G6-P respectively.)

No. of rats	O ₂ consumption (μ l.)		Increase in O ₂ consumption in the presence of G1-P (μ l.)
	Glucose	G1-P	
8	209 $\pm$ 6.6	324 $\pm$ 17.4	115 $\pm$ 16.7 $P=0.01$
3	230 $\pm$ 10.0	217 $\pm$ 14	

Table 9. *Influence of the ionic concentration on the insulin effect on glycogenesis*

(The diaphragms were incubated for 90 min. at 37° in 3 ml. of medium, containing glucose (2%) and 1 unit insulin/ml. Gas phase, O₂. Mean values given $\pm$ S.E.M.)

No. of rats	Initial glycogen (mg./g. wet wt.) tissue	NaCl concn. of medium (M)	Increase in glycogen (mg./g. wet wt.) tissue
8	0.69	0.08	0.44* $\pm$ 0.09
4	0.66	0.20	0.44 $\pm$ 0.02

* Results taken from Table 8.

an initial glycogen content of 0.88 mg./g. (mean value) was no significant insulin effect observed in the presence of glucose. With G1-P there was no significant effect of insulin in any group. The substrates were used in a concentration of 2%, as at lower concentration there was little or no synthesis of glycogen from G1-P (Verzár & Wenner, 1948).

In order to ascertain that the difference in the effect of insulin on glycogen synthesis in systems containing glucose and G1-P, respectively, was not due to the increased ionic concentration of the medium in the latter, some experiments were carried out with glucose in a medium containing an increased sodium chloride concentration (0.20M) so that the ionic conditions closely approximated

It might be suggested that the addition of sodium phosphate to the medium, instead of sodium chloride, would have created ionic conditions more closely resembling those existing in the presence of the sodium salt of the phosphate ester; this, however, was not feasible owing to the inhibitory effect of high concentrations of inorganic phosphate on glycogen synthesis, a phenomenon described in experiments with rat diaphragm muscle by Stadie & Zapp (1947), and confirmed by the present authors.

Oxygen consumption. The results of these experiments are shown in Table 10. There was a considerably higher oxygen consumption by the diaphragm

Table 11. *Effect of 2:4-dinitrophenol (DNP) on oxygen consumption by rat diaphragm muscle in presence of glucose and G 1-P*

(Results expressed as $\mu\text{l. O}_2/100 \text{ mg. wet wt. of diaphragm}/90 \text{ min. at } 37^\circ \text{ in O}_2$. Mean values given $\pm \text{s.e.m.}$ Concentration of DNP = $4.83 \times 10^{-5} \text{ M}$. A positive or negative effect of DNP indicates an increase or decrease in O_2 uptake, respectively. The hemidiaphragms from each rat were incubated with and without DNP respectively.)

No. of rats	Substrate (2%)	O ₂ uptake ($\mu\text{l.}$)		Effect of DNP
		No DNP	With DNP	
9	Glucose	170 $\pm$ 10	342 $\pm$ 13.4	+ 172 $\pm$ 16.6 $P = < 0.01$
9	G 1-P	273 $\pm$ 8.9	168 $\pm$ 8.7	- 105 $\pm$ 12.7 $P = < 0.01$

muscle incubated with G 1-P compared with that incubated with glucose. The addition of glucose to the medium had no effect on the respiration of diaphragm muscle.

The effect of 2:4-dinitrophenol on respiration. Another interesting contrast in the behaviour of glucose and G 1-P is the effect of DNP on the respiration of rat diaphragm muscle in the presence of these substrates. The results (Table 11) show that in the presence of glucose, in confirmation of the findings of Pierce & Field (1949), the oxygen consumption was greatly increased by DNP, whereas in the presence of G 1-P a considerable inhibition was observed.

The metabolism of G 6-P and F 6-P

Equilibrium between G 6-P and F 6-P. It was shown that in rat diaphragm muscle there is a very active phosphohexoisomerase enzyme, described by Lohmann (1933) in muscle suspensions, and by

Table 12. *Isomerization of F 6-P and G 6-P by rat diaphragm muscle*

(Rat diaphragm muscle incubated for 30 min. at 37° in presence of F 6-P or G 6-P. Mean values given $\pm \text{s.e.m.}$)

No. of rats	Substrate and concentration	F 6-P disappearing from medium (%)	G 6-P converted into F 6-P (%)
		(%)	(%)
3	2% G 6-P	—	44 $\pm$ 0.55
3	2% F 6-P	62 $\pm$ 2.65	—
3	0.2% G 6-P	—	47 $\pm$ 1.0
3	0.2% F 6-P	60 $\pm$ 2.52	—

Wajzer (1951) in haemolysed blood, which acts on F 6-P or G 6-P forming an equilibrium mixture of the two phosphate esters containing approximately 40% F 6-P and 60% G 6-P. The transformation of one ester into the other was measured either by the disappearance of F 6-P when added to the medium, or by the formation of F 6-P when G 6-P was the added substrate (Table 12). The total phosphorus of the medium was not found to alter during incubation, and this suggested that there was an isomerization of one phosphate ester into the other. This was

confirmed by paper chromatography according to the method of Hanes & Isherwood (1949).

The phosphohexoisomerase was found to be active at room temperature and the equilibrium was established in about 15 min. The enzyme appeared to diffuse out readily from the muscle. Thus it was found that by washing the diaphragm muscle for 15 min. in 3 ml. of medium, an active extract of the isomerase was obtained, and on repeating this extraction three times with three lots of medium, the isomerase enzyme was active in each extract and the diaphragm itself still retained some activity.

In the following experiments on oxygen consumption and glycogen synthesis, 2% G 6-P was the added substrate; however, owing to the rapid formation of an equilibrium mixture by the isomerase enzyme, the medium in fact contained approximately 0.8% F 6-P and 1.2% G 6-P.

Oxygen consumption. The results of these experiments are given in Table 10, and show that there is no increase in oxygen consumption in the presence of G 6-P as added substrate.

Glycogen synthesis. The results given in Table 13 show that in the absence of insulin a glycogenolysis occurs when diaphragm muscle is incubated in an equilibrium mixture of G 6-P and F 6-P. In the presence of insulin glycogenolysis was inhibited in most experiments, and in some a little glycogen was synthesized.

The metabolism of fructosediphosphate

As shown in Table 13, there was no synthesis of glycogen in the presence of 2% F 1:6-P. No direct comparison was made of the respiration in the presence of glucose and F 1:6-P with hemidiaphragms of the same rats; however the oxygen uptake observed in the presence of 2% F 1:6-P was of the same order as that found in other experiments with 2% glucose as substrate.

DISCUSSION

In vivo experiments. The results reported in the present paper bring out two main points. First, contrary to what has been suggested in the literature

Table 13. *Change in glycogen content of rat diaphragm muscle incubated with G 6-P with and without insulin or F 1:6-P without insulin*

(The diaphragms were incubated for 90 min. at 37° in 3 ml. of medium in O₂. Mean values given $\pm$ s.e.m. Substrates, 2%. Insulin, 1 unit/ml. medium. A negative change indicates glycogenolysis.)

No. of rats	Initial glycogen content (mg./g. wet wt.) diaphragm		Added substrate	Change in glycogen content (mg./g. wet wt.)		Insulin effect	P
	Limiting values	Mean values		No insulin	With insulin		
8	0.20-0.59	0.32	G 6-P	-0.02 $\pm$ 0.01	+0.19 $\pm$ 0.01	0.21 $\pm$ 0.01	0.10
8	0.60-1.00	0.82	G 6-P	-0.07 $\pm$ 0.09	+0.22 $\pm$ 0.06	0.29 $\pm$ 0.10	0.10
9	1	1.32	G 6-P	-0.12 $\pm$ 0.05	+0.07 $\pm$ 0.07	0.19 $\pm$ 0.10	0.02
4	0.4-0.8	0.66	F 1:6-P	-0.44 $\pm$ 0.10	—	—	—

(Conway, 1942; Verzá & Wenner, 1948), the hexose phosphates can penetrate into the tissue cells and are metabolized. The rapid disappearance of intravenously injected G 1-P and G 6-P from the blood, which cannot be accounted for by a phosphatase activity in the blood or by a quantitative excretion of the esters in the urine, can only be explained by the passage of the esters from the blood and extracellular spaces into the tissue cells. The subsequent increases in blood glucose and inorganic phosphorus in the blood and urine supply strong evidence that the esters are metabolized in the cells, although no evidence is available as to which tissues are concerned.

It is impossible to estimate from our results what proportion of the injected phosphate esters are acted on by the tissue phosphatases. However, if the sole metabolic pathway of the esters is the hydrolysis to glucose and inorganic phosphorus, a consistently greater increase in the blood glucose of the diabetic rabbits, particularly in those experiments with animals in which the ureters were ligated, might be expected.

The work of Roberts & Wolffe (1951) is of interest as regards the question of permeability of the hexose phosphate esters into living cells. Using F 6-P and F 1:6-P labelled with ³²P, these authors report that the cells of *Escherichia coli* are readily permeable to these esters.

The findings that about 30% of the G 1-P and 40-50% of the G 6-P injected are excreted in the urine are of interest in so far as they indicate that the two phosphate esters must be transferred intact through the glomerular membrane, or less likely through the tubules.

The second main point of interest arising from the *in vivo* experiments is that both G 6-P and G 1-P, in contrast to glucose, appear to have a normal tolerance curve in the alloxan-diabetic rabbit, and excretion of these esters was essentially the same in normal and diabetic animals. These findings can be interpreted as additional evidence for the hypothesis that at least one block in carbohydrate metabolism

in the diabetic animal occurs in the phosphorylation of glucose.

In vitro experiments. The first main observation of interest arising from these experiments with rat diaphragm muscle was the absence of a positive insulin effect on substrate uptake and glycogen synthesis in the presence of G 1-P in contrast to that observed in the presence of glucose. This observation is consistent with the theory that insulin is concerned with a preliminary phosphorylation of glucose. This theory, however, cannot account for the apparently inhibitory effect of insulin on G 1-P uptake and the authors at present have no explanation for this effect. The increased oxygen consumption by rat diaphragm muscle in the presence of G 1-P, and the action of DNP on the systems studied, can also not be explained at present.

The second important result in these experiments was the fundamental difference observed between the behaviour of G 1-P and G 6-P in the presence of rat diaphragm muscle. G 6-P was shown to form rapidly an equilibrium mixture with F 6-P, and diaphragm muscle incubated in this mixture neither synthesized glycogen nor showed an increase in oxygen consumption. In fact, G 6-P was found to be relatively inert in striking contrast to G 1-P. These observations are not consistent with the generally accepted theories of glucose metabolism, in which it is assumed that G 6-P is always the first intermediate.

SUMMARY

1. It has been demonstrated that the hexose phosphates can penetrate into the tissue cells of rabbits, and evidence is advanced that they are metabolized within the cells.

2. In contrast to the behaviour of glucose, intravenously injected glucose 1-phosphate and glucose 6-phosphate disappeared rapidly from the blood of both normal and alloxan-diabetic rabbits; a subsequent increase in blood glucose and in the inorganic phosphorus of the blood and urine was observed.

3. Approximately 30% of the injected glucose 1-phosphate was recovered in the urine of both normal and alloxan-diabetic rabbits and 40 and 55% of the injected glucose 6-phosphate in the urine of normal and diabetic rabbits, respectively.

4. In the presence of isolated rat diaphragm muscle, the uptake of glucose 1-phosphate from the medium was higher than that of glucose. Addition of insulin increased the glucose uptake and inhibited the uptake of glucose 1-phosphate.

5. The amount of glycogen synthesis by rat diaphragm muscle was of the same order in systems containing glucose 1-phosphate (2%) and systems containing glucose (2%), and in both cases was largely dependent on the initial glycogen content of the muscle. Insulin increased the glycogen synthesis from glucose but had no effect on glycogen synthesis from glucose 1-phosphate.

6. Oxygen consumption of isolated rat diaphragm was increased by the addition of glucose 1-phosphate (2%) and unaffected by glucose. When 2:4-dinitrophenol ($4.83 \times 10^{-5} M$) was added to the medium, oxygen consumption was increased in the presence of glucose and inhibited in the presence of glucose 1-phosphate.

7. Glucose 6-phosphate and fructose 6-phosphate were acted on by an isomerase enzyme present in rat diaphragm muscle forming an equilibrium mixture containing approximately 40% fructose 6-phosphate and 60% glucose 6-phosphate. Rat diaphragm muscle incubated in this equilibrium mixture did not synthesize glycogen nor was its oxygen consumption affected.

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