the α - and β -globin chains of haemoglobin which are not yet in the form of the finished haemoglobin molecule.

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The Metabolism of Glucose 1-Phosphate and Glucose 6-Phosphate and their Influence on the Metabolism of Glucose in Rat-Diaphragm Muscle

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In a previous investigation carried out in this Laboratory (Beloff-Chain *et al.* 1953) it was found that isolated rat diaphragm synthesized glycogen from glucose and from glucose 1-phosphate to about the same extent when the substrate concentration,

calculated as glucose, was 2%. There was little or no glycogen synthesis from glucose 6-phosphate at this concentration. Further, it was shown that insulin had no stimulatory effect on glycogen synthesis from glucose 1-phosphate as it had from glucose.

It was decided to try to confirm these findings by using ^{14}C -labelled glucose 1-phosphate and glucose 6-phosphate and to follow the entire pattern of metabolism of the hexose phosphate esters in rat diaphragm by applying the technique of quantitative radiochromatography, which has been previously applied to the study of glucose metabolism in diaphragm muscle (Beloff-Chain *et al.* 1955). The influence of glucose 6-phosphate and glucose 1-phosphate on glucose metabolism in the isolated diaphragm was also studied by applying the same technique.

EXPERIMENTAL

Animals. Male Wistar rats weighing approx. 200 g. and starved for 24 hr. before an experiment were used.

Materials. ^{14}C -labelled glucose 6-phosphate was prepared according to the method developed in this Laboratory (Betto & Longinotti, 1963).

^{14}C -labelled glucose and ^{14}C -labelled starch were obtained from The Radiochemical Centre, Amersham, Bucks.

^{14}C -labelled glucose 1-phosphate was prepared from 50 mg. of ^{14}C -labelled starch (specific activity $15\ \mu\text{C}/\text{mg.}$) treated with potato phosphorylase (prepared by the method of McCready & Hassid, 1957). The procedure for the enzymic phosphorylase of starch to glucose 1-phosphate was that described by Baroncelli, Betto & Munoz Gil (1960). The deproteinized aqueous solution containing glucose 1-phosphate was diluted to 50 ml. and the glucose 1-phosphate was isolated and purified by the following procedure. The solution was run through a 30 ml. column (12 cm. diam. $\times$ 27 cm.) of Dowex 1 (X8; Cl^- form; 200–400 mesh) at 0.5 ml./min. The unchanged starch was eluted from the resin with 300 ml. of mN-NH_4 solution. Then 1.4 l. of a solution containing NH_4Cl (25 mM) and $\text{K}_2\text{B}_4\text{O}_7$ (10 mM) was passed through the column at 1 ml./min., and fractions (15 ml.) were collected by using an automatic fraction collector. The fractions containing ^{14}C glucose 1-phosphate (a radioactive peak eluted before glucose 1-phosphate was discarded) were pooled and passed through another 75 ml. column (1.7 cm. diam. $\times$ 33 cm.) of Dowex 1 (X8; formate form; 200–400 mesh) at 1 ml./min. This column was washed with 200 ml. of mN -formic acid to eliminate traces of borate and to produce an acid condition in the resin, and the glucose 1-phosphate was then eluted with 0.7N-formic acid passed through the column at 2 ml./min. The fractions containing glucose 1-phosphate were then pooled and concentrated under reduced pressure at a temperature not exceeding 10° . The remaining formic acid was removed by repeatedly redissolving the residue in water and concentrating *in vacuo* and finally by extracting three times with ether. The ether having been removed the residue was dissolved in water to give the required volume and the pH adjusted to 6.8 with NaOH . The final radioactive product was shown to be chromatographically pure glucose 1-phosphate, free from formic acid, and by an enzymic determination of glucose 1-phosphate the specific activity was found to be the same as that of the original starch.

Incubation of diaphragm muscle. Substrate concentration is expressed throughout as glucose concentration.

The conditions of incubation used were the same as those described by Beloff-Chain *et al.* (1955). In all experiments

the muscle was divided into four parts of approx. 50 mg. In the first series of experiments two of these were incubated with ^{14}C glucose, in the absence and presence of insulin respectively, and two with ^{14}C -labelled hexose phosphate ester, in the absence and presence of insulin respectively. The substrate concentration was 1% (w/v); $30\ \mu\text{C}$ was added/vessel.

The influence of glucose 6-phosphate (1%) on glucose metabolism in the absence and presence of insulin was studied by incubating each part of the diaphragm with 0.1% ^{14}C glucose ($10\ \mu\text{C}$), one part without additions, one part in the presence of non-radioactive glucose 6-phosphate (1%), one part in the presence of insulin and one part in the presence of insulin and glucose 6-phosphate.

The influence of non-radioactive glucose 6-phosphate at different concentrations was studied by incubating each part of the diaphragm in the presence of 0.1% ^{14}C glucose ($10\ \mu\text{C}$), one part in the absence of glucose 6-phosphate and the other three parts in the presence of 0.01, 0.1 and 1% glucose 6-phosphate respectively.

The influence of glucose 1-phosphate on glucose metabolism was investigated by incubating the four pieces of diaphragm muscle with 0.1% ^{14}C glucose ($10\ \mu\text{C}$), two in the absence and two in the presence of glucose 1-phosphate (1%).

Preparation of samples for radioactive measurements. The methods of preparation of tissue extracts after incubation and the chromatography methods were identical with those described by Beloff-Chain *et al.* (1955). The quantitative recovery of the ^{14}C radioactivity added to the medium was discussed by Beloff-Chain *et al.* (1955).

The radiochromatograms were scanned by the automatic scanning device developed in this Laboratory (Frank, Chain, Pocchiari & Rossi, 1959).

RESULTS

Metabolism of glucose, glucose 1-phosphate and glucose 6-phosphate. Qualitatively there was no difference in the pattern of metabolism of the hexose phosphate esters and of glucose. Thus the radioactive material disappearing from the medium could be accounted for as glycogen, oligosaccharides, lactate and carbon dioxide. In all experiments the 'zone g' of the radiochromatograms contained mostly unchanged substrate, but no attempt has yet been made to separate out from this zone other intermediates that may be present in small amounts, and which have similar R_f values in the solvents used to those of the substrates.

Quantitatively (Table 1) the three substrates were metabolized very differently. Thus under the experimental conditions used in the present work the synthesis of glycogen and oligosaccharides from glucose 1-phosphate was considerably greater than from glucose, whereas there was much less synthesis of glycogen from glucose 6-phosphate than from glucose. The concentration of unchanged substrate in the tissue was considerably greater with glucose than with glucose 1-phosphate. The concentration of substrate in the tissues indicates

the substrate remaining after three washings of the muscle in 0.9 % sodium chloride. It was previously shown, with glucose as substrate, that the concentration of substrate in the tissue as defined above was proportional to the external concentration in the range 0.1–1 % (Beloff-Chain, Catanzaro, Chain, Masi & Pocchiari, 1956). The formation of lactate was about the same from glucose and glucose 6-phosphate, but somewhat less from glucose 1-phosphate, whereas the formation of carbon dioxide was higher from both the phosphate esters than from glucose.

The results given in Table 1 also show that insulin had no effect on the metabolism of the phosphate esters. The well-known effect of insulin on glycogen synthesis from glucose was observed, and there was a small but statistically significant increase in zone *g* and diminution of lactate formation from glucose in the presence of insulin.

Influence of glucose 6-phosphate and glucose 1-phosphate on glucose metabolism. The results given in Table 2 show that glucose 6-phosphate in a concentration of 1 % considerably increased the incorporation of radioactivity from glucose (0.1 %) into glycogen and oligosaccharides, as well as increasing the glucose concentration in the tissue. This effect of glucose 6-phosphate was observed both in the absence and presence of insulin. Lactate

formation from glucose was considerably decreased by the presence of glucose 6-phosphate.

To ascertain whether these effects were obtained only in the presence of a high concentration of glucose 6-phosphate relative to the glucose concentration, another series of experiments were carried out in the presence of 0.01 %, 0.1 % and 1 % glucose 6-phosphate. The results given in Table 3 show that a significant effect of glucose 6-phosphate on the incorporation of ^{14}C from glucose into glycogen and oligosaccharides was apparent only in the presence of a high concentration (1 %) of glucose 6-phosphate; however, at low concentration there was an effect on the formation of radioactive lactate.

The results given in Table 4 show that glucose 1-phosphate also slightly increased the incorporation of ^{14}C from glucose into glycogen and oligosaccharides. As with glucose 6-phosphate the formation of lactate from glucose was decreased.

From the results reported in Tables 2 and 4 it would appear that glucose 6-phosphate decreased the formation of $^{14}\text{CO}_2$ from $[^{14}\text{C}]$ glucose, whereas glucose 1-phosphate slightly increased it. However, these effects were not consistently reproducible, and considerable variations were found in the results obtained in later experiments in which only $^{14}\text{CO}_2$ formation was measured.

Table 1. *Influence of insulin on the metabolism of glucose, glucose 1-phosphate and glucose 6-phosphate in rat-diaphragm muscle*

Results are expressed as μm -moles of substrate converted and of unchanged substrate/50 mg. of tissue after incubation for 90 min. in 0.6 ml. of phosphate-buffered medium, pH 6.8, at 37° in O_2 . The substrate concentration was 1 % (expressed as glucose) (approx. $30 \mu\text{C}/\text{vessel}$); the insulin concentration was 0.1 unit/ml. Mean values $\pm$ s.e.m. are given, with the numbers of experiments in parentheses. Zone *g* in this and the other Tables is mostly unchanged substrate (see the text).

	Glucose (19)		Glucose 1-phosphate (11)		Glucose 6-phosphate (8)	
	No insulin	With insulin	No insulin	With insulin	No insulin	With insulin
Glycogen and oligosaccharides	236.1 $\pm$ 21.4	460.9* $\pm$ 64.7	410.8 $\pm$ 27.9	412.7 $\pm$ 21.5	61.3 $\pm$ 8.1	53.8 $\pm$ 6.9
Zone <i>g</i>	1316.5 $\pm$ 71.7	1593.1* $\pm$ 70.2	464.4 $\pm$ 23.3	443.4 $\pm$ 30.9	705.3 $\pm$ 43.6	775.1 $\pm$ 80.8
Lactate	865.4 $\pm$ 39.0	708.2* $\pm$ 51.8	556.7 $\pm$ 27.4	526.5 $\pm$ 24.1	840.9 $\pm$ 100.6	843.2 $\pm$ 69.1
Carbon dioxide	124.9 $\pm$ 12.4	132.4 $\pm$ 9.6	217.0 $\pm$ 12.0	219.7 $\pm$ 13.0	291.2 $\pm$ 35.8	324.1 $\pm$ 26.6

* Difference from the result in the absence of insulin significant, i.e. $P < 0.01$.

Table 2. *Influence of glucose 6-phosphate and insulin on the metabolism of glucose in rat-diaphragm muscle*

Results are expressed in μm -moles of glucose converted and of unchanged glucose/50 mg. of tissue after incubation for 90 min. in 0.6 ml. of phosphate-buffered medium, pH 6.8, at 37° in O_2 . The concentration of $[^{14}\text{C}]$ glucose was 0.1 % (approx. $10 \mu\text{C}/\text{vessel}$); the non-radioactive glucose 6-phosphate concentration was 1 % (expressed as glucose); the insulin concentration was 0.1 unit/ml. The mean values of four experiments $\pm$ s.e.m. are given.

	Glucose		Glucose + glucose 6-phosphate	
	No insulin	With insulin	No insulin	With insulin
Glycogen and oligosaccharides	66.4 $\pm$ 25.0	208.2 $\pm$ 39.9	124.8 $\pm$ 19.9	253.8 $\pm$ 51.5
Zone <i>g</i>	98.9 $\pm$ 14.9	135.8 $\pm$ 15.3	166.6 $\pm$ 4.9	190.2 $\pm$ 6.2
Lactate	700.7 $\pm$ 62.5	621.0 $\pm$ 24.0	308.8 $\pm$ 41.9	328.5 $\pm$ 43.7
Carbon dioxide	209.7 $\pm$ 35.9	229.9 $\pm$ 13.6	120.5 $\pm$ 14.0	129.6 $\pm$ 7.0

Table 3. *Influence of glucose 6-phosphate at different concentrations on the metabolism of glucose in rat-diaphragm muscle*

Results are expressed in μ m-moles of glucose converted and of unchanged glucose under the experimental conditions given in Table 2. Mean values of four experiments $\pm$ S.E.M. are given.

Concn. of glucose 6-phosphate (%) ...	0	0.01	0.1	1.0
Glycogen and oligosaccharides	48.1 $\pm$ 11.9	48.9 $\pm$ 6.6	42.3 $\pm$ 10.1	67.3 $\pm$ 6.8
Zone <i>g</i>	114.3 $\pm$ 9.1	126.9 $\pm$ 8.7	128.5 $\pm$ 9.4	172.2 $\pm$ 2.2
Lactate	428.8 $\pm$ 46.9	384.3 $\pm$ 27.3	347.8 $\pm$ 29.6	292.4 $\pm$ 20.1
Carbon dioxide	89.9 $\pm$ 8.8	86.4 $\pm$ 13.3	72.0 $\pm$ 4.5	85.8 $\pm$ 5.9

Table 4. *Influence of glucose 1-phosphate on the metabolism of glucose in rat-diaphragm muscle*

Results are expressed as μ m-moles of [14 C]glucose converted and of unchanged glucose under the experimental conditions given in Table 2. The non-radioactive glucose 1-phosphate concentration was 1% (expressed as glucose). Mean values of eight experiments $\pm$ S.E.M. are given.

	Glucose	Glucose + glucose 1-phosphate
Glycogen and oligo-saccharides	41.1 $\pm$ 6.9	56.7 $\pm$ 8.6*
Zone <i>g</i>	138.3 $\pm$ 4.9	149.1 $\pm$ 11.4
Lactate	432.6 $\pm$ 30.9	271.1 $\pm$ 14.1*
Carbon dioxide	66.5 $\pm$ 5.1	81.2 $\pm$ 10.5

* Difference from the result with glucose alone significant, i.e. $P < 0.05$.

DISCUSSION

The use of the usual isolated rat-diaphragm preparation has been severely criticized by some authors on the ground that most of the muscle fibres in such preparations are damaged by cutting. As a much preferable alternative the use of the so-called 'intact' preparation of Kipnis & Cori (1957) has been advocated. The present authors have used isolated diaphragm preparations throughout their work, and intend to do so in the future, so that they deem it appropriate to comment briefly on the controversy of 'cut' versus 'intact' diaphragm.

It is possible that the intact preparation (Kipnis & Cori, 1957) may have some advantages over the isolated preparations for the study of permeability of metabolically inert sugars, though all effects so far described as occurring in intact preparations were also clearly observed in cut preparations, if quantitatively less pronounced. But, whatever the merits of the intact-diaphragm preparations for permeability studies of inert sugars, the present authors consider it useless for the study of metabolic processes in the rat diaphragm, because in such preparations the weight of the diaphragm part constitutes only about 10% of the total, and the rest consists of extraneous tissues, such as cut intercostal muscle and bone. This mass of non-diaphragm tissue not only meta-

bolizes added substrate, but exudes large quantities of proteins, among them enzymes of the glycolytic cycle, into the incubation medium. These may act on the substrate present in the incubation medium, as in fact they do in the present case (unpublished work), where nearly half of the added glucose 1-phosphate is converted in the incubation medium into a mixture of glucose 6-phosphate and fructose 6-phosphate, a phenomenon that does not occur with the isolated diaphragm, and which obscures, of course, the metabolic fate of these substrates in the diaphragm muscle proper.

The results reported in the present paper confirm the earlier conclusion from this Laboratory that glucose 1-phosphate is readily synthesized to glycogen in the isolated rat-diaphragm muscle incubated for 90 min., whereas glucose 6-phosphate is only very poorly converted into glycogen. As it is generally assumed that glucose 1-phosphate is formed from glucose 6-phosphate, it is difficult to interpret this difference in behaviour of these two hexose phosphate esters in the light of our present knowledge. It seems very unlikely that the explanation can be a difference of penetration of these two substances into the tissue, because there is probably a higher concentration of unchanged substrate in the tissue after incubation with glucose 6-phosphate than with glucose 1-phosphate, and, further, the former produces more lactate and carbon dioxide. The mechanism of synthesis of glycogen from glucose 1-phosphate is not yet clarified and could possibly be a reversal of phosphorylase activity. The possibility was considered that an excess of glucose 6-phosphate might inhibit the enzyme phosphoglucomutase and thus prevent the formation of glucose 1-phosphate and glycogen. Experiments were therefore carried out in which glucose 6-phosphate (1%) was added to a medium containing 0.1% of [14 C]glucose, to ascertain whether under these conditions glycogen synthesis was inhibited. However, the results showed that glucose 6-phosphate stimulated glycogen synthesis from glucose, whereas it diluted the radioactive lactate, as would be expected.

The most obvious explanation for the stimulation of glycogen synthesis by glucose 6-phosphate would be that the latter is an activator of glycogen

synthetase (Leloir, Olavarria, Goldemberg & Carminatti, 1959). However, this property of glucose 6-phosphate would not account for its increasing the concentration of glucose in the tissue; further, it would be expected that, if glucose 6-phosphate were acting as an activator of glycogen synthetase, the effect would be observed at low concentrations. The results presented above show that only in the presence of a relatively high concentration (1%) of glucose 6-phosphate was there a significant effect on glycogen synthesis and concurrently an effect on glucose concentration in the tissue.

The experiments carried out in the presence of insulin and glucose 6-phosphate showed that their stimulatory effects on glycogen synthesis from glucose were approximately additive and therefore independent of each other. It appears probable that the mechanism of action of glucose 6-phosphate on glycogen synthesis from glucose is, in part at least, correlated with its effect on increasing the entry of glucose into the tissue, whereas the mechanism of insulin action on glycogen synthesis in diaphragm muscle, as was shown by Beloff-Chain *et al.* (1956), cannot be explained in this way.

The effect of non-radioactive glucose 1-phosphate in a medium containing [^{14}C]glucose was different from that of glucose 6-phosphate. Glucose 1-phosphate produced a less marked increase in the incorporation of [^{14}C]glucose into glycogen and had no influence on the concentration of [^{14}C]glucose in the tissue.

The results reported above cannot be adequately explained by our present knowledge of the mechanism of glycogen synthesis in muscle, but suggest that glucose 6-phosphate is not converted into glucose 1-phosphate and is not on the pathway of glycogen synthesis from glucose. It is of interest in this connexion that Figueroa, Pfeiffer & Niemeyer (1962), as a result of experiments with liver homogenates, made the tentative suggestion that 'the reaction glucose $\rightarrow$ glucose 6-phosphate is not a necessary step in glycogen synthesis'.

The reason for the variable results obtained in experiments in which the influence of the hexose phosphate esters on the formation of carbon dioxide from glucose was measured is not yet clear; however, the oxidation of glucose by diaphragm muscle accounts for only about 2% of the total carbon dioxide produced. Therefore small biological variations in the use of glucose as substrate by different pieces of diaphragm muscle would result in considerable differences in the $^{14}\text{CO}_2$ produced.

SUMMARY

1. The pattern of metabolism of [^{14}C]glucose 1-phosphate and [^{14}C]glucose 6-phosphate has been followed in rat-diaphragm muscle by applying a quantitative radiochromatographic technique. The hexose phosphate esters, like glucose, were converted into glycogen, oligosaccharides, lactate and carbon dioxide.

2. With a substrate concentration of 1% (w/v) (calculated as glucose) glycogen synthesis was greater from glucose 1-phosphate than from glucose, whereas the synthesis from glucose 6-phosphate was much less. Lactate formation was about the same from glucose and glucose 6-phosphate, but less from glucose 1-phosphate. Carbon dioxide formation was higher from the hexose phosphate esters than from glucose.

3. Insulin had no influence on the metabolism of the hexose phosphate esters.

4. Glucose 6-phosphate (1%) increased glycogen synthesis from [^{14}C]glucose (0.1%) and increased the [^{14}C]glucose concentration in the tissue, in the absence and presence of insulin. These effects were not observed in the presence of lower concentrations (0.01 and 0.1%) of glucose 6-phosphate. Glucose 6-phosphate in concentrations of 0.01, 0.1 and 1% decreased lactate formation from 0.1% [^{14}C]glucose.

5. Glucose 1-phosphate (1%) only slightly increased glycogen synthesis from [^{14}C]glucose and decreased lactate formation from glucose.

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