EFFECT OF PHOSPHATE AND BICARBONATE IONS ON THE FATE OF GLUCOSE-1-PHOSPHATE AND GLUCOSE IN ISOLATED RAT DIAPHRAGM MUSCLE

by

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In a previous publication from this laboratory (BELOFF-CHAIN, CHAIN, BOVET, POCCHIARI, CATANZARO AND LONGINOTTI[1]) results of experiments on the metabolism of hexose phosphate esters and of glucose in isolated rat diaphragm muscle incubated in a phosphate saline medium were reported. These investigations have been extended by studying the influence of the anionic composition of the incubation medium on the metabolism of these substrates.

STADIE AND ZAPP[2] reported that varying the phosphate concentration from 0 to 0.05 *M* did not influence glycogen synthesis in the isolated rat diaphragm muscle but that at higher phosphate concentrations synthesis was slightly depressed. These results were confirmed by VILLEE, DEANE AND HASTINGS[3], who also reported an increased glucose uptake at high phosphate concentrations (0.08 *M* and 0.160 *M*).

After the completion of the present work the paper of ZIERLER, LEVY AND ANDRES[4] came to the attention of the authors. In this paper it has been shown that glucose-1-phosphate incubated in a potassium phosphate buffered medium is converted by rat diaphragm muscle into a mixture of hexose-phosphates consisting probably of glucose-6-phosphate, fructose-6-phosphate and fructose-1-6-phosphate; some lactic acid formation was also shown to occur. The glycolytic enzymes causing the dissimilation of glucose-1-phosphate were shown to be present in a cell free effluent of the muscle. These authors have reported that no glycogen synthesis occurs in the presence of glucose-1-phosphate, a finding which can be accounted for by the fact that the experiments were carried out in the presence of low concentrations of substrate (25–50 μM per 3 ml of medium) and confirms our own findings previously published (BELOFF-CHAIN *et al.*[1]).

In the present work experiments were carried out on the metabolism of glucose-1-phosphate, glucose-6-phosphate and glucose by isolated rat diaphragm muscle incubated in phosphate, bicarbonate/CO_2 and tris(hydroxymethyl)aminomethane buffers.

METHODS AND MATERIAL

Preparations. Glucose-1-phosphate (G-1-P) and glucose-6-phosphate (G-6-P) and their sodium salts were prepared as previously described (BELOFF-CHAIN *et al.*[1]).

Analytical methods. Glucose was determined by the method of MILLER AND VAN SLYKE[5].

P was determined using the procedure of BERENBLUM AND CHAIN[6].

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Glycogen was determined using the procedure of GOOD, KRAMER AND SOMOGYI[7] as modified by WALAAS AND WALAAS[8].

G-1-P was determined by measuring the inorganic phosphorus present before and after 10 minutes hydrolysis in N H_2SO_4 at 100°.

Fructose-phosphate ester was determined according to the method of ROE[9].

Oxygen consumption was measured by the usual Warburg manometric technique.

PROCEDURE

The experimental procedure was similar to that previously described (BELOFF-CHAIN *et al.*[1]).

Male rats of the Wistar strain weighing 100–180 g were killed by stunning and decapitation. The diaphragms (weighing about 250 mg each) were rapidly removed, divided into two, three or four approximately equal parts, each of which was weighed and transferred to 3 ml of chilled medium at pH 6.8–7.0, in a Warburg vessel. In all experiments pieces of diaphragm from the same rat were incubated in two or three of the media with the following composition:

Bicarbonate medium. 0.01 M $NaHCO_3$, 0.11 M NaCl, 0.005 M $MgCl_2$. A mixture of 95% O_2 — 5% CO_2 was passed through the medium to adjust the pH before the addition of the muscle, and again after the flasks were attached to the manometers.

Phosphate medium. (STADIE AND ZAPP[2]) 0.04 M Na_2HPO_4, 0.08 M NaCl, 0.005 M $MgCl_2$. The medium was oxygenated for 5 min.

Tris medium. 0.016 M Tris(hydroxymethyl)aminomethane (GOMORI[10]). 0.104 M NaCl, 0.005 M $MgCl_2$. The medium was oxygenated for 5 min.

Glycogen synthesis. The procedure was identical to that previously employed (BELOFF-CHAIN *et al.*[1]) except that the pieces of diaphragm were digested in 0.3 ml, instead of 2 ml, of 30% KOH.

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

RESULTS

Glycogen analysis

It will be seen in the results given below that the values for glycogen synthesis reported in the present work which were determined by the method of WALAAS AND WALAAS[8] were much higher than those given in a previous publication (BELOFF-CHAIN *et al.*[1]), in which the method of STADIE, HAUGAARD AND MARSH[11] was adopted. It was therefore decided to compare these two methods of glycogen analysis in the following way. A rat diaphragm was divided into approximately four equal parts in two of which the glycogen content was determined by the method of STADIE *et al.*[10] (referred to below as macro-method) and of WALAAS AND WALAAS[8] (micro-method) respectively. The remaining two pieces of diaphragm muscle were incubated in the presence of 2% glucose in a phosphate medium using the procedure described above. At the end of the incubation period the glycogen content of the two pieces was determined by the macro- and micro-method respectively. The results of these experiments carried out with ten rats are given in Table I and show that the micro-method gives slightly higher results for the initial glycogen content of the muscle, but the discrepancy between the two methods is very much greater in measuring the glycogen present after incubation of the muscle with glucose.

This finding is of interest as it shows a marked difference in the behaviour of the glycogen present initially in the diaphragm muscle and that formed after incubation with glucose.

Duplicate analysis of the glycogen content of pieces of diaphragm muscle before and after incubation with glucose did not differ by more than 10% using either method of analysis. This was shown in experiments on six rats by the method of STADIE *et al.*[11] and on 8 rats by the method of WALAAS AND WALAAS[8].

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TABLE I

GLYCOGEN CONTENT OF RAT DIAPHRAGM MUSCLE AS DETERMINED BY THE METHODS OF STADIE *et al.*[11] AND WALAAS AND WALAAS[8]

Rat diaphragm muscle incubated for 90 min at 37° in 3 ml of medium. Mean values given ± S.E.M. Substrate concentration 2%. – Macro-method indicates method of STADIE *et al.*[11]. – Micro-method indicates method of WALAAS AND WALAAS[8].

No. of rats	*Initial glycogen content of diaphragm (mg/100 mg wet wt.) tissue*		*Glycogen content of diaphragm after incubation with glucose (mg/100 mg wet wt.) tissue*	
	Macro method	*Micro method*	*Macro method*	*Micro method*
10	0.09 ± 0.005	0.108 ± 0.005	0.157 ± 0.008	0.261 ± 0.015

Fate of G-1-P

Substrate disappearance. It was found that when G-1-P (0.2%)* was incubated with 100 mg rat diaphragm muscle for 90 min at 37° in the bicarbonate medium, approximately 70% of the substrate originally present (6 mg) disappeared. Experiments carried out previously (BELOFF-CHAIN *et al.*[1]), showed that only about 12% of the substrate disappeared under the above conditions in the phosphate medium.

Formation of fructose-phosphate ester from G-1-P. The results of these experiments are given in Table II. The term fructose phosphate-ester is used to denote the substance measured by the method of ROE[9], as no attempt was made to determine the nature of this ester. It appears probable according to the findings of ZIERLER *et al.*[4] that a mixture of fructose-6-phosphate and fructose-1-6-phosphate is formed. The results show that the accumulation of fructose phosphate ester was much greater in the bicarbonate and tris medium than in the phosphate medium. These findings could be explained by the observation of CORI, COLOWICK AND CORI[12] that phosphate buffer inhibits the enzyme phosphoglucomutase, which catalyzes the conversion of G-1-P to G-6-P in tissue extracts (CORI, COLOWICK AND CORI[13]). As was shown previously (BELOFF-CHAIN *et al.*[1]) G-6-P rapidly forms an equilibrium mixture with F-6-P in the presence of rat diaphragm muscle, consisting of approximately 40% F-6-P and 60% G-6-P. It would appear therefore from these results that the greatly increased disappearance of G-1-P (0.2%)

TABLE II

INFLUENCE OF THE COMPOSITION OF THE INCUBATION MEDIUM ON THE FORMATION OF FRUCTOSE-PHOSPHATE ESTER FROM G-1-P BY ISOLATED RAT DIAPHRAGM MUSCLE

(Rat diaphragm muscle (approximately 100 mg wet wt.) was incubated for 90 min at 37° in 3 ml of medium. Mean values ± S.E.M.)

No. of rats	*Substrate concentration*	*Fructose phosphate ester formed in medium (mg/100 mg wet wt.) tissue*		
		Tris medium	*Bicarbonate medium*	*Phosphate medium*
8	0.2	—	1.43 ± 0.22	0.39 ± 0.03
9	2	—	1.80 ± 0.16	0.76 ± 0.12
5	2	1.24 ± 0.06	—	0.62 ± 0.10

* The concentrations of the hexose phosphate esters are expressed throughout as mg of glucose or fructose present.

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in the presence of diaphragm muscle suspended in a bicarbonate medium as compared with a phosphate medium can be accounted for by the increased accumulation of other hexose-phosphate esters. Increasing the concentration of G-1-P from 0.2% to 2% only caused a relatively small increase in fructose-phosphate ester formation in the bicarbonate medium. This indicates that the amount of enzyme present for this conversion is the limiting factor under the conditions of these experiments.

Phosphoglucomutase appears to diffuse out readily from the diaphragm into the medium, as was shown previously in the case of phosphohexoisomerase (BELOFF-CHAIN *et al.*[1]). Thus by shaking the diaphragm muscle for 30 min in 3 ml of bicarbonate medium, an active extract of phosphoglucomutase could be obtained which, on incubating with G-1-P (2%), showed approximately 70% of the activity of the intact diaphragm.

Glycogen synthesis. The results given in Table III show that in the absence of added phosphate ions, *i.e.* in the bicarbonate medium or tris medium, there was little glycogen synthesis from 2% G-1-P, as compared with the synthesis observed in the phosphate medium.

Insulin did not effect glycogen synthesis from G-1-P in any of the three media studied.

TABLE III

INFLUENCE OF THE COMPOSITION OF THE INCUBATION MEDIUM ON GLYCOGENESIS FROM G-1-P BY ISOLATED RAT DIAPHRAGM MUSCLE

(Rat diaphragm muscle (approximately 100 mg wet wt.) incubated for 90 min at 37° in 3 ml of medium. Mean values given ± S.E.M. Substrate concentration 2%. Glycogen synthesis indicates the increase in glycogen over the initial value.)

No. of rats	*Initial glycogen content of diaphragm (mg/100 mg wet wt.) tissue*	*Glycogen synthesis (mg/100 mg wet wt.) tissue*		
		Tris medium	*Bicarbonate medium*	*Phosphate medium*
12	0.14 ± 0.01	—	0.096 ± 0.01	0.36 ± 0.02
5	0.16 ± 0.02	0.076 ± 0.02	—	0.24 ± 0.02

Oxygen consumption. It was shown previously (BELOFF-CHAIN *et al.*[1]) that G-1-P (2%) increases the oxygen consumption of isolated rat diaphragm muscle suspended in a phosphate buffer. From the results given in Table IV it is evident that in the presence of G-1-P (2%) the oxygen consumption is the same in the phosphate or tris medium, and in both cases is significantly higher than the oxygen uptake in the presence of glucose (2%).

TABLE IV

OXYGEN CONSUMPTION BY RAT DIAPHRAGM MUSCLE IN THE PRESENCE OF G-1-P AND GLUCOSE

(Results expressed as μl O_2/100 mg wet weight of diaphragm in 90 min at 37° in O_2. Mean values given ± S.E.M. Substrate concentration 2%.)

No. of rats	*Substrate*	*Oxygen consumption (μl)*	
		Phosphate medium	*Tris medium*
5	G-1-P	285 ± 25	295 ± 27
5	Glucose	186 ± 5.7	169 ± 9.7

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Fate of glucose

Substrate disappearance. From the results given in Table V, it is evident that the disappearance of glucose (0.2%) was significantly higher in bicarbonate and slightly higher in tris than in phosphate.

TABLE V

INFLUENCE OF THE COMPOSITION OF THE INCUBATION MEDIUM ON THE UTILIZATION OF GLUCOSE BY ISOLATED RAT DIAPHRAGM MUSCLE

(Rat diaphragm muscle incubated for 90 min at 37° in 3 ml of medium. Mean values given ± S.E.M.)

No. of rats	*Glucose disappearing from medium (mg/100 mg wet wt.) tissue*		
	Bicarbonate medium	*Tris medium*	*Phosphate medium*
9	1.62 ± 0.20	1.01 ± 0.06	0.80 ± 0.07

Glycogen synthesis. The results of these experiments are given in Table VI. Experiment 1 shows that the glycogen synthesis from glucose was much higher in the bicarbonate medium than in the phosphate medium*.

TABLE VI

INFLUENCE OF THE COMPOSITION OF THE INCUBATION MEDIUM ON GLYCOGENESIS FROM GLUCOSE BY ISOLATED RAT DIAPHRAGM MUSCLE

(Rat diaphragm muscle incubated for 90 min at 37° in 3 ml of medium. Mean values given ± S.E.M. Substrate concentration 2%. Glycogen synthesis indicates the increase in glycogen over initial value.)

Experiment	*No. of rats*	*Initial glycogen content of diaphragm (mg/100 mg wet wt.) tissue*	*Glycogen synthesis (mg/100 mg wet wt.) tissue*		
			Tris medium	*Bicarbonate medium*	*Phosphate medium*
1	12	0.11 ± 0.015	—	0.63 ± 0.07	0.29 ± 0.04
2	5	0.065 ± 0.01	0.32 ± 0.01	—	0.22 ± 0.03
3	5	0.093 ± 0.03	0.40 ± 0.02	0.56 ± 0.02	0.27 ± 0.03

In Experiment 2 in which the muscle was incubated in the tris and phosphate medium respectively, synthesis was again lower in the latter, but the difference was very much less marked. These findings were confirmed in Experiment 3 in which the diaphragm was divided into four approximately equal parts; one of which was utilized for the determination of the initial glycogen content and the remaining three of which were incubated in the bicarbonate, tris and phosphate medium respectively.

In two experiments the influence of insulin on glycogen synthesis from glucose in phosphate and bicarbonate medium respectively was measured, the results are given in Table VII. It appears that the medium has no striking influence on the insulin effect.

* These results have been confirmed in an experiment (to be reported elsewhere) in which diaphragm muscle was incubated in phosphate and bicarbonate medium respectively in the presence of uniformly labelled ^{14}C glucose, and the radioactivity of the isolated glycogen was determined.

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TABLE VII

THE INFLUENCE OF THE COMPOSITION OF THE INCUBATION MEDIUM ON THE INSULIN EFFECT ON GLYCOGENESIS FROM GLUCOSE BY ISOLATED RAT DIAPHRAGM MUSCLE

(Rat diaphragm muscle incubated for 90 min at 37° in 3 ml of medium. Substrate concentration 2%. Glycogen synthesis indicates the increase in glycogen content over the initial value. Each line represents the results of an experiment carried out with the diaphragm of one rat.)

Experiment	*Medium*	*Glycogen synthesis (mg/100 mg wet wt.) tissue*	
		No insulin	*With insulin*
1	Phosphate	0.26	0.48
		0.33	0.47
		0.10	0.15
2	Bicarbonate	0.38	0.61
		0.51	0.87
		0.52	0.64

Fate of G-6-P

It was found that the composition of the medium did not effect the conversion of G-6-P into F-6-P by rat diaphragm muscle or by an extract of the muscle; thus an equilibrium mixture of these two esters was formed in the bicarbonate medium under the same conditions as previously described in the phosphate medium (BELOFF-CHAIN *et al.*[1]).

As can be seen from the results given in Table VIII, a glycogenolysis occurs when diaphragm muscle is incubated in the presence of G-6-P (2%) either in the phosphate or bicarbonate medium.

TABLE VIII

CHANGE IN GLYCOGEN CONTENT OF RAT DIAPHRAGM MUSCLE INCUBATED WITH G-6-P

(Rat diaphragm muscle (approximately 100 mg wet wt.) incubated for 90 min at 37° in 3 ml of medium. Mean values given ± S.E.M. Substrate concentration 2%. A negative change in glycogen content indicates glycogenolysis.)

No. of rats	*Initial glycogen content of diaphragm (mg/100 mg wet wt.) tissue*	*Change in glycogen content (mg/100 mg wet wt.) tissue*	
		Bicarbonate medium	*Phosphate medium*
6	0.18 ± 0.03	−0.09 ± 0.03	−0.12 ± 0.01

DISCUSSION

From the results reported in the present paper it is evident that optimal glycogen synthesis in isolated rat diaphragm muscle from G-1-P requires the presence of phosphate ions in the incubation medium while optimal glycogen synthesis from glucose was obtained in the absence of added phosphate ions. Thus phosphate ions have the opposite effect on glycogen synthesis in rat diaphragm muscle from G-1-P and glucose respectively. One possible explanation, but not the only one, for the influence of phosphate ions on the fate of G-1-P is that phosphate inhibits the conversion of G-1-P

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to G-6-P by the phosphoglucomutase enzyme and thus directs the pathway of its metabolism towards glycogen synthesis. As it has been shown that G-1-P increases oxygen uptake by rat diaphragm muscle to the same extent with and without phosphate ions in the medium, it does not appear that the penetration of this substrate into the cells is prevented in the absence of phosphate ions.

The fact that G-1-P forms very little glycogen in the absence of added phosphate ions whereas glucose is readily transformed to glycogen under these conditions is difficult to reconcile with the generally accepted theory that all glycogen synthesis must proceed via G-1-P, and the results reported in the present work suggest that there may be an alternative pathway for glycogen synthesis from glucose in the muscle.

Another point of interest arising out of the present work, is that both the synthesis of glycogen from glucose and glucose utilization by rat diaphragm muscle are significantly augmented in the presence of the bicarbonate medium. This is of particular interest in view of the findings of VENNESLAND, SOLOMON, BUCHANAN AND HASTINGS[14], that when glucose was fed to rats followed by injections of a solution of $NaH^{11}CO_3$, radioactive carbon was found in appreciable quantities both in muscle and liver glycogen. LORBER, HEMINGWAY AND NIER[15] have also reported that CO_2 is incorporated into glycogen synthesised from glucose by an isolated heart.

Studies on glucose metabolism by rat diaphragm muscle in the presence of radioactive bicarbonate are now in progress and will be reported in a subsequent publication.

SUMMARY

1. When glucose-1-phosphate was incubated with isolated rat diaphragm muscle in a bicarbonate or tris buffered medium there was a greater accumulation of fructose-phosphate ester in the medium and a lower synthesis of glycogen than in a phosphate buffered medium.

2. The utilization of glucose and the synthesis of glycogen from glucose by rat diaphragm muscle was increased considerably in a bicarbonate and slightly in a tris buffered medium as compared with the phosphate buffered medium.

3. A glycogenolysis was observed in rat diaphragm muscle incubated with glucose-6-phosphate regardless of whether the medium was buffered with bicarbonate or phosphate.

RÉSUMÉ

1. Quand du glucose-1-phosphate est incubé en présence de muscle du diaphragme isolé du rat, l'accumulation de l'ester fructose-phosphorique est plus importante et la synthèse du glycogène plus faible dans un milieu tamponné par du bicarbonate ou du "tris", que dans un milieu tamponné par un phosphate.

2. L'utilisation du glucose et la synthèse du glycogène à partir du glucose par le muscle du diaphragme du rat sont plus élevées dans un tampon "tris" et surtout dans un tampon bicarbonate que dans un tampon phosphate.

3. Le muscle du diaphragme du rat incubé avec du glucose-6-phosphate est le siège d'une glycogénolyse, quel que soit le tampon (bicarbonate ou phosphate) employé.

ZUSAMMENFASSUNG

1. Wenn Glucose-1-Phosphat mit isoliertem Diaphragmamuskel der Ratte in Bicarbonat- oder Tris-Puffer inkubiert wurde, wurde mehr Fructose-phosphatester in dem Medium angehäuft und weniger Glykogen synthetisiert als in einem Phosphatpuffer.

2. Der Glucoseverbrauch und die Glykogensynthese aus Glucose durch den Diaphragmamuskel der Ratte waren, im Vergleich zu einem mit Phosphat gepufferten Medium, in einem mit Bikarbonat gepufferten Medium bedeutend, in einem mit Tris gepufferten Medium ein wenig erhöht.

3. Im Diaphragmamuskel der Ratte konnte bei Inkubation mit Glucose-6-phosphat Glykogenolyse beobachtet werden und zwar sowohl in einem mit Bikarbonat als in einem mit Phosphat gepufferten Medium.

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Received February 10th, 1954