Influence of some Mononucleotides and their corresponding Nucleosides on the Metabolism of Carbohydrates in the Isolated Rat Diaphragm Muscle

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1. The influence of ATP on glucose metabolism was studied in the isolated rat diaphragm; it was shown that ATP increases the oxidation of glucose and the aerobic conversion of glucose into lactate, whereas it decreases glycogen synthesis. There was no influence of ATP on the anaerobic formation of lactate from glucose. 2. A maximum effect of ATP on the oxidation of glucose (about 160% increase) was obtained in the presence of 10mm-ATP; in the presence of 2mm-ATP the effect was about 65%, and was approximately constant from 10 to 90min. incubation period. 3. In a phosphate-free tris-buffered medium the oxidation of glucose was considerably decreased, but the percentage stimulation by ATP was about the same as in a phosphate-buffered medium. 4. ATP was shown to increase the oxidation of fructose, glucose 6-phosphate, glucose 1-phosphate, fructose 1,6-diphosphate and, to a much smaller extent, pyruvate. 5. ADP stimulated the oxidation of glucose to the same extent as ATP at a concentration of 2mm and the effect with AMP was only slightly less; IMP and adenosine had only a small stimulatory effect at this concentration, whereas inosine had no effect.

During studies on carbohydrate metabolism in the isolated rat diaphragm carried out in this Laboratory, it was observed that the addition of ATP to the incubation medium increased the oxidation of glucose. It was decided to investigate this observation in detail and to study the effect of ATP on the complete pattern of glucose metabolism in the isolated diaphragm and its effect on the oxidation of fructose, glucose 1-phosphate, glucose 6-phosphate, fructose 1,6-diphosphate and pyruvate. It has been shown by Alertsen, Walaas & Walaas (1958) that ATP is rapidly broken down by diaphragm muscle to form ADP, AMP, IMP and inosine; the effect of these nucleotides and the corresponding nucleosides on the oxidation of glucose was also studied in the present work. The results of these investigations are reported below.

EXPERIMENTAL

Animals. Male Wistar rats weighing approx. 150g. and starved for 24hr. before an experiment were used.

Materials. [^{14}C]Glucose, [^{14}C]pyruvate and [^{3-14}C]pyruvate were obtained from The Radiochemical Centre,

Amersham, Bucks. [^{14}C]Glucose 6-phosphate and [^{14}C]glucose 1-phosphate were prepared according to the methods reported by Betto & Longinotti (1963) and Beloff-Chain *et al.* (1964). [^{14}C]Fructose 1,6-diphosphate was obtained from Schwarz BioResearch Inc., Mount Vernon, N.Y., U.S.A.

Incubation of diaphragm muscle. The preparation of the isolated diaphragm and the conditions of incubation were as described by Beloff-Chain *et al.* (1955). The two hemidiaphragms were each divided into two approximately equal parts, each weighing about 50mg. Each part was incubated in 0.6 ml. of incubation medium in small Warburg vessels (with a total volume of 7–8 ml.). Unless otherwise stated a phosphate-buffered medium at pH 6.8 was used (Stadie & Zapp, 1947), containing Na_2HPO_4 (40 mm), NaCl (80 mm) and MgCl_2 (5 mm). In those experiments in which tris-buffered medium replaced the phosphate medium the composition of the former was tris, pH 6.8 (16 mm), NaCl (104 mm) and MgCl_2 (5 mm). Conditions of incubation under anaerobic conditions were as described by Beloff-Chain *et al.* (1959).

In those experiments in which the influence of ATP on the metabolism of glucose and other substrates was investigated, pieces taken from the same diaphragm muscle were incubated in the absence and presence of ATP. In those experiments in which the influence of phosphate on the ATP effect on glucose metabolism was studied, two pieces of muscle were incubated in the phosphate-buffered medium and two in the tris-buffered medium, in each case without and with ATP respectively.

The influence of different nucleotides and nucleosides on glucose oxidation was studied by using one piece of the muscle in each experiment as a control, incubated with

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[U-¹⁴C]glucose; the remaining pieces were incubated with [U-¹⁴C]glucose and the nucleotides or nucleosides under investigation as indicated in Table 5.

The influence of increasing ATP concentration on the oxidation of glucose was followed by incubating one piece of diaphragm without ATP and the remaining pieces with the different concentrations of ATP studied as shown in Fig. 1.

In the experiments in which the influence of time on the ATP effect was studied, ATP (2mm) was present in all the vessels and three pieces of muscle from one diaphragm were incubated for 10, 30 and 90 min. respectively.

Preparation of samples for radioactive measurements. The method of preparation of aqueous-ethanolic tissue extracts after incubation, the sampling of the incubation medium for measurements of radioactive intermediates, the treatment of the insoluble tissue residue after extraction, and the collection and measurement of ¹⁴CO₂ were as described by Beloff-Chain *et al.* (1955). The radiochromatograms were scanned by the automatic scanning device developed in this Laboratory (Frank, Chain, Pocchiari & Rossi, 1959).

The results for lactate are the sum of that present in the extract and the incubation medium; glycogen was determined as the sum of that in the soluble and insoluble fractions; oligosaccharides were all present in the ethanolic tissue extracts.

Calculation of results. The initial radioactivity of the incubation medium, i.e. of the added glucose, was measured in each experiment; thus the counts/min./μmole of added glucose could be calculated. The radioactivity of the different substances measured was then calculated in terms of the μmoles of added glucose converted. The size of the glucose pool in the tissues is regarded as being of no significance in these calculations.

RESULTS

Influence of ATP on the metabolism of glucose. As has been shown by Beloff-Chain *et al.* (1955), glucose is converted *in vitro* in the isolated diaphragm muscle into glycogen [part of which is soluble after extraction with aq. 60% (v/v) ethanol, and part of which remains in the residue after extraction], oligosaccharides, lactate (which is present both in the tissue extract and the incubation medium) and carbon dioxide. Some unchanged glucose was always recovered in the tissue extracts.

The results given in Table 1 show that ATP decreased the incorporation of glucose into glycogen

and oligosaccharides, whereas it increased the conversion into lactate (the values given are the sum of that present in the extract and the incubation media) and carbon dioxide.

By increasing the ATP concentration from 2mm to 10mm the effect on ¹⁴CO₂ formation was augmented from about a 65% increase to nearly a 160% increase (Fig. 1). The increase in ¹⁴CO₂ formation from [U-¹⁴C]glucose produced by the addition of ATP was apparent already after 10 min. incubation (Fig. 2). The percentage increase was approximately constant over a period of 90 min., as shown in Fig. 2.

Under anaerobic conditions the amount of lactate formed from glucose was, as reported by Beloff-Chain *et al.* (1959), much decreased, and this was not affected by the addition of ATP to the medium (Table 2). The remainder of the glucose disappearing from the medium under anaerobic conditions was recovered as free unchanged glucose in the tissue, and the concentration of the latter was also unaffected by ATP (Table 2).

Fig. 1. Influence of ATP at various concentrations on the oxidation of glucose by rat diaphragm muscle incubated *in vitro*. Experimental conditions were as given in Table 1. Results are expressed as the percentage increase of ¹⁴CO₂ formation from [U-¹⁴C]glucose in the presence of various concentrations of ATP over that formed in the absence of ATP. The results are the means of five experiments.

Table 1. Influence of ATP on the metabolism of glucose by rat diaphragm muscle incubated *in vitro*

Results are expressed as mμmoles of glucose converted and of unchanged glucose/50 mg. of tissue after 90 min. incubation at 37° in O₂ in 0.6 ml. of phosphate-buffered medium, pH 6.8, containing glucose (5.5 mm; total radioactivity 10 μC/vessel) with or without ATP (2.0 mm). Mean values ± s.e.m. of six experiments are given. 'Zone g' represents mostly unchanged glucose, and 'insoluble residue' represents mainly glycogen insoluble under the conditions used for tissue extraction.

	Glycogen + oligosaccharides	Zone g	Lactate	CO ₂	Insoluble residue
Without ATP	12.8 ± 1.1	92.2 ± 9.4	512.2 ± 19.4	121.6 ± 9.4	15.0 ± 2.2
With ATP	7.8 ± 1.7*	86.7 ± 10.5	710.5 ± 46.6*	202.2 ± 6.1	10.0 ± 1.1

* Difference significant ($P < 0.01$).

Incubation of the muscle in a phosphate-free medium, buffered with tris, caused a considerable decrease in the oxidation of glucose without affecting oxygen uptake, but ATP stimulated the oxidation of glucose also in the absence of phosphate

(Table 3). There was no significant effect of ATP on oxygen consumption either in the absence or presence of phosphate.

Influence of ATP on the oxidation of hexose phosphate esters and pyruvate. Results given in Table 4 show that ATP increased the oxidation of fructose, glucose 6-phosphate, glucose 1-phosphate and fructose 1,6-diphosphate, whereas there was only a small effect on the oxidation of pyruvate. This latter effect was only evident when $[3-^{14}\text{C}]$ -pyruvate was used; with $[1-^{14}\text{C}]$ pyruvate, where the $^{14}\text{CO}_2$ formed was much greater than with $[3-^{14}\text{C}]$ pyruvate, no effect of ATP was observed. The effect of ATP on the oxidation of fructose 1,6-diphosphate (about 300%) was much greater than on that of glucose (about 70%).

Influence of some nucleotides and their corresponding nucleosides on the oxidation of glucose. The results given in Table 5 show that ADP had the same stimulatory effect (about 65%) as ATP on the oxidation of glucose at a concentration of 2mM, and the effect of AMP was only slightly lower (about 50%). IMP and adenosine also slightly increased the oxidation of glucose (about 30%), whereas inosine had no effect.

Fig. 2. Glucose converted into CO_2 at various time-periods in the absence and presence of ATP by rat diaphragm muscle incubated *in vitro*. Experimental conditions were as given in Table 1. Incubations were performed in the absence (○) and in the presence (●) of ATP (2mM). The results are the means of three experiments.

DISCUSSION

When ATP was added to an incubation medium containing glucose, oxidation of the latter by the isolated rat diaphragm muscle was greatly increased. This effect of ATP was independent of whether the incubation medium contained phosphate or not, although omitting phosphate from the medium greatly decreased the oxidation of glucose. Further, it was shown that the aerobic formation of lactate from glucose was also stimulated by ATP (whereas the anaerobic lactate formation was not affected) and the incorporation of glucose into glycogen was decreased. The influence of ATP on the oxidation of glucose appears to be a rapid effect. It was already observed after 10min. incubation, and the percentage increase in $^{14}\text{CO}_2$ formation remained almost constant over a 90min. period of incubation.

It cannot be concluded from the present investigation whether the ATP effect observed was due

Table 2. *Influence of ATP on the anaerobic metabolism of glucose to lactate in rat diaphragm muscle incubated in vitro*

Experimental conditions and results are expressed as in Table 1. Mean values $\pm$ S.E.M. of five experiments are given.

	Zone g	Lactate
Without ATP	209.2 $\pm$ 8.3	112.8 $\pm$ 11.6
With ATP	208.1 $\pm$ 8.9	135.0 $\pm$ 28.3

Table 3. *Influence of the composition of the incubation medium on the oxygen consumption and oxidation of glucose by rat diaphragm muscle incubated in vitro in the absence and presence of ATP*

Results are expressed as μmoles of O_2 and $^{14}\text{CO}_2/50\text{mg.}$ of tissue after 90min. incubation at 37° in O_2 in 0.6ml. of medium, pH 6.8, containing glucose (5.5mM; total radioactivity $10\mu\text{C/vessel}$) with or without ATP (2.0mM). Mean values $\pm$ S.E.M. of six experiments are given.

Incubation medium	$^{14}\text{CO}_2$ production		O_2 uptake	
	Without ATP	With ATP	Without ATP	With ATP
Phosphate-buffered	1.00 $\pm$ 0.06	1.54 $\pm$ 0.08	5.7 $\pm$ 0.3	6.1 $\pm$ 0.4
Tris-buffered	0.35 $\pm$ 0.04	0.75 $\pm$ 0.06	5.4 $\pm$ 0.2	6.1 $\pm$ 0.3

Table 4. *Influence of ATP on oxygen uptake and $^{14}\text{CO}_2$ production from glucose, fructose, hexose phosphate esters and pyruvate in rat diaphragm muscle incubated in vitro*

Results are expressed as μmoles of O_2 and $^{14}\text{CO}_2/50\text{ mg.}$ of tissue after 90 min. incubation at 37° in O_2 in 0.6 ml. of phosphate-buffered medium, pH 6.8, containing $\text{U-}^{14}\text{C}$ -labelled glucose, fructose 1,6-diphosphate, glucose 6-phosphate or glucose 1-phosphate (5.5 mm) or $[\text{3-}^{14}\text{C}]$ - or $[\text{1-}^{14}\text{C}]$ -pyruvate (11.1 mm) with or without ATP (2.0 mm); $10\text{ }\mu\text{C}$ of radioactive substrate was added/vessel. Mean values $\pm$ s.e.m. are given, with the numbers of experiments in parentheses.

Substrate	$^{14}\text{CO}_2$ production		O_2 uptake	
	Without ATP	With ATP	Without ATP	With ATP
$[\text{U-}^{14}\text{C}]$ Glucose (12)	0.70 ± 0.08	1.20 ± 0.13	5.8 ± 0.3	6.1 ± 0.2
$[\text{U-}^{14}\text{C}]$ Fructose (3)	0.60 ± 0.03	1.00 ± 0.02	5.5 ± 0.1	5.7 ± 0.2
$[\text{U-}^{14}\text{C}]$ Glucose 6-phosphate (2)	0.36	1.60	5.0	5.0
$[\text{U-}^{14}\text{C}]$ Glucose 1-phosphate (2)	0.30	0.83	5.0	5.1
$[\text{U-}^{14}\text{C}]$ Fructose 1,6-diphosphate (6)	0.50 ± 0.05	2.03 ± 0.11	6.6 ± 0.3	7.0 ± 0.2
$[\text{1-}^{14}\text{C}]$ Pyruvate (4)	2.10 ± 0.12	2.20 ± 0.07	6.4 ± 0.3	6.6 ± 0.3
$[\text{3-}^{14}\text{C}]$ Pyruvate (9)	0.65 ± 0.03	0.88 ± 0.01		

Table 5. *Influence of some nucleosides and nucleotides on $^{14}\text{CO}_2$ production from glucose by rat diaphragm muscle incubated in vitro*

Results are expressed as amounts of glucose converted into $^{14}\text{CO}_2/50\text{ mg.}$ of tissue after 90 min. incubation at 37° in O_2 , in 0.6 ml. of phosphate-buffered medium, pH 6.8, containing $[\text{U-}^{14}\text{C}]$ glucose (5 mm; total radioactivity $10\text{ }\mu\text{C}$ /vessel) with or without nucleoside or nucleotide (2 mm). The $^{14}\text{CO}_2$ produced from glucose in the absence of nucleosides and nucleotides was taken to be equal to 100. Mean values $\pm$ s.e.m. are given, with the numbers of experiments in parentheses.

Expt. no.	Addition	$^{14}\text{CO}_2$ production
I	None	(100)
	ATP	161 ± 15 (11)
	IMP	133 ± 17 (11)
	Inosine	100 ± 10 (11)
II	None	(100)
	ATP	167 ± 12 (8)
	Adenosine	133 ± 8 (8)
III	None	(100)
	ATP	160 ± 15 (9)
	ADP	156 ± 18 (9)
	AMP	145 ± 11 (9)

in fact to the intact ATP molecule or to the presence of ADP or AMP, which are rapidly formed from ATP, probably at the muscle cell surface (Alertsen *et al.* 1958), and which have been shown to have a similar effect.

The enzymic site of the ATP effect also remains to be elucidated, but as the stimulation of the oxidation of fructose 1,6-diphosphate was even greater than that of glucose it is clearly not a direct action on the phosphorylation of glucose by the hexokinase reaction.

Further investigations will be required to establish whether ATP itself or one of its degradation products is responsible for this very marked effect on carbohydrate metabolism in muscle, and to ascertain the enzymic site of action.

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