Effect of Atractyloside on Glucose and Pyruvate Metabolism in Rat Diaphragm Muscle

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1. In the presence of 1.2 mM-atractyloside oxygen uptake by rat diaphragm muscle incubated with 5.6 mM-glucose decreases, as well as glycogen synthesis and carbon dioxide production. Lactate formation from glucose increases, but that of phosphoglycerate diminishes fivefold. 2. When pyruvate is used as substrate, atractyloside decreases oxygen uptake. 3. The specific radioactivity of the $^{14}\text{CO}_2$ ($\mu\text{moles of } ^{14}\text{CO}_2/\mu\text{mole of oxygen}$), calculated at concentrations of $[1\text{-}^{14}\text{C}]\text{pyruvate}$ between 0.091 mM and 91 mM, lies between 3.1×10^{-4} and 5.7×10^{-1} . Atractyloside increases the specific radioactivity of the $^{14}\text{CO}_2$ with the lowest concentrations of substrate and has no effect when the substrate concentration is 91 mM. 4. No appreciable effect of atractyloside on the anaerobic production of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{pyruvate}$ at various incubation times and various concentrations is found. 5. It is suggested that atractyloside induces anaerobic conditions in the tissue. Further, it produces a rise in the pyruvate concentration and an ATP deficiency in the cell. Consequently it stimulates pyruvate dismutation, and glycolysis, to which phosphorylation is linked at the substrate level.

Atractyloside is highly poisonous to all animals with the exception of invertebrates (Tocco, 1922). The action of the glucoside on mitochondria has been widely studied (Kemp & Slater, 1964; Chappell & Crofts, 1965; Bruni, Luciani & Bortignon, 1965; Brierley & O'Brien, 1965; Vignais, Duee, Vignais & Huet, 1966; Moret, Lorini, Fotia & Siliprandi, 1966; Charles & Van den Bergh, 1967), but apart from the work of Bruni, Rausa & Patania (1958) little is known about its action on the intact tissue. For this reason it seemed to us useful to extend the research *in vitro* to various animal tissues. The present paper gives some quantitative data on the effect of atractyloside on glucose and pyruvate metabolism in rat diaphragm muscle. Preliminary data have been reported (Pocchiari, Silano & Santi, 1966).

METHODS AND MATERIALS

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

Materials. $[\text{U-}^{14}\text{C}]\text{Glucose}$, $[1\text{-}^{14}\text{C}]\text{glucose}$, $[6\text{-}^{14}\text{C}]\text{glucose}$, $[\text{U-}^{14}\text{C}]\text{pyruvate}$, $[1\text{-}^{14}\text{C}]\text{pyruvate}$ and $[3\text{-}^{14}\text{C}]\text{pyruvate}$ were obtained from The Radiochemical Centre, Amersham, Bucks.; $[3,4\text{-}^{14}\text{C}_2]\text{glucose}$ was obtained from the New England Nuclear Corp., Boston, Mass., U.S.A.

Incubation of diaphragm muscle. The conditions of incubation were the same as those described by Beloff-Chain *et al.* (1955).

Approx. 100 mg. of diaphragm muscle was incubated in

1.2 ml. of a phosphate-buffered medium (Stadie & Zapp, 1947) at pH 6.8. The medium was equilibrated for 5 min. in O_2 or in N_2 ; in anaerobic experiments substrate was added from the side arm of the Warburg vessel after equilibration with N_2 .

Determination of $^{14}\text{CO}_2$ production at various incubation times. In these experiments the rat diaphragm muscle was divided into four parts, one being incubated for 30 min. and the remaining three for periods of 60, 120 and 180 min. in the phosphate-buffered medium at pH 6.8 and at 37° (in O_2 or N_2) with substrate.

Unless otherwise stated, the glucose and pyruvate concentrations were 5.6 mM and 9.1 mM respectively; $20 \mu\text{C}$ was added to each vessel. The atractyloside concentration was 1.2 mM.

Preparation of samples for tissue radioactivity measurements. The methods of preparation of tissue extracts after incubation and the methods of chromatography were those described by Beloff-Chain *et al.* (1955). The pieces of diaphragm muscle were removed from the Warburg vessels, blotted on filter paper and rapidly washed in three lots of 0.9% NaCl solution and blotted on filter paper after each washing. The muscle was then transferred to 2 ml. of 60% (w/v) ethanol and homogenized in a small Potter homogenizer, and transferred to a centrifuge tube; the homogenizer was washed with 1 ml. of 60% (v/v) ethanol and the washing was added to the muscle homogenate. The latter was centrifuged and the ethanolic extract collected, the insoluble residue being re-extracted once with 1 ml. of 60% (v/v) ethanol and twice with 1 ml. of water. The extracts were pooled and evaporated to dryness in a rotary evaporator (Buchler Instruments, New York, N.Y., U.S.A.). The dried extract was dissolved in 0.5 ml. of water.

Paper chromatography. Samples (50 μ l.) of the extract and samples (20 μ l.) of the incubation medium were chromatographed on Whatman no. 1 filter paper. The following solvents mixtures were used: solvent 1, picric acid-butan-1-ol-water (4:8:1, w/v/v); solvent 2, butan-1-ol-acetic acid-water (40:11:25, by vol.); solvent 3, phenol-water-aq. ammonia (sp.gr. 0.88) (80:20:1, by vol.); solvent 4, butan-1-ol-formic acid (85%, w/v)-water (15:3:2, by vol.).

Oligosaccharides, glucose and phosphoglycerate were separated by one-dimensional chromatography with solvent 1, lactate was detected by one-dimensional chromatography with solvent 2 and the amino acids were separated by two-dimensional chromatography with solvent 3 followed by solvent 4.

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

The $^{14}\text{CO}_2$ radioactivity was determined by the liquid-scintillation technique. CO_2 was trapped on a filter-paper strip wetted with 0.2 ml. of 0.4 N-NaOH. The paper strip was plunged into 3 ml. of water. After 1 hr. 1 ml. of the mixture was added to 10 ml. of Bray's (1960) solution and counted in a Nuclear-Chicago Mk. 1 liquid-scintillation counter with an efficiency of about 60%.

The insoluble residue was transferred to aluminium planchets, dried under an infrared lamp and counted under a mica-window Geiger counter.

Total glycogen was obtained by the procedure described by Shaw & Stadie (1957) and hydrolysed to glucose with $\text{N-H}_2\text{SO}_4$. After neutralization of the hydrolysate, radioactivity was determined in the liquid-scintillation counter.

RESULTS

Glucose metabolism. Atractyloside decreased oxygen uptake in the rat diaphragm muscle as had already been observed, in the absence of glucose in the incubation medium, by Bruni *et al.* (1958). The decrease was about 25% with the glucoside present at a concentration of 1.2 mM. Therefore this concentration was chosen as a working condition.

The inhibitory effect of the glucoside became more pronounced as the incubation time was increased; thus during incubation for 5 hr. oxygen uptake without atractyloside rose almost linearly, but that in the presence of the glucoside was progressively decreased. The production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]\text{glucose}$ was influenced in the same way (Fig. 1).

Table 1 shows that in the presence of atractyloside the production of $^{14}\text{CO}_2$ by rat diaphragm muscle from glucose labelled with ^{14}C in different carbon atoms was decreased. In preliminary experiments (Pocchiari *et al.* 1966) it was found that atractyloside stimulated $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glucose}$. At the moment we have no explanation for these differences.

Atractyloside did not change the qualitative

Fig. 1. Effect of atractyloside on O_2 uptake and $^{14}\text{CO}_2$ production in rat diaphragm muscle at various incubation times. Tissue (100 mg.) was incubated in 1.2 ml. of phosphate-buffered medium, pH 6.8, at 37° in O_2 . The glucose concentration was 5.6 mM and that of atractyloside, when present, was 1.2 mM. $\circ$, O_2 uptake without atractyloside; $\triangle$, O_2 uptake with atractyloside; $\bullet$, $^{14}\text{CO}_2$ production without atractyloside; $\blacktriangle$, $^{14}\text{CO}_2$ production with atractyloside.

Table 1. Influence of atractyloside on $^{14}\text{CO}_2$ production in rat diaphragm muscle from glucose labelled with ^{14}C in different positions

Incubation was for 90 min. in 1.2 ml. of phosphate-buffered medium, pH 6.8, at 37° in O_2 . The glucose concentration was 5.6 mM (approx. 20 μC /vessel); the atractyloside concentration was 1.2 mM. Mean values $\pm$ S.E.M. are given with the numbers of experiments in parentheses.

Substrate	Glucose converted into CO_2 ($\mu\text{g.}/100\text{ mg. of tissue}$)	
	No atractyloside	With atractyloside
$[\text{U-}^{14}\text{C}]\text{Glucose}$ (7)	34 ± 4	30 ± 4
$[\text{1-}^{14}\text{C}]\text{Glucose}$ (14)	28 ± 3	23 ± 3
$[\text{6-}^{14}\text{C}]\text{Glucose}$ (17)	25 ± 4	23 ± 4
$[\text{3,4-}^{14}\text{C}_2]\text{Glucose}$ (12)	42 ± 4	35 ± 3

pattern of glucose metabolism in rat diaphragm, nor the total quantity of metabolized glucose (i.e. the total radioactivity recovered in the individual metabolites; see Beloff-Chain *et al.* 1955). On the other hand the glucoside considerably altered the distribution of the radioactivity of the substrate among the various metabolites (Table 2). Production of radioactive lactate was significantly raised whereas that of phosphoglycerate and glycogen was diminished. These effects of atractyloside became more evident when the incubation time was doubled and glucose concentration halved (Table 3).

If the specific radioactivity of glucose was taken

Table 2. Influence of atractyloside on [U-¹⁴C]-glucose metabolism in rat diaphragm muscle

Experimental conditions were as described in Table 1. The methods of preparation of tissue extracts after incubation and the methods of chromatography were those described by Beloff-Chain *et al.* (1955). Mean values of ten experiments $\pm$ s.e.m. are given.

	Glucose converted (μ g./100 mg. of tissue)	
	No atractyloside	With atractyloside
Glycogen and oligo- saccharides	24 $\pm$ 5	18 $\pm$ 3
Zone g*	29 $\pm$ 3	31 $\pm$ 3
Lactate	188 $\pm$ 24	268 $\pm$ 59
Phosphoglycerate	64 $\pm$ 12	12 $\pm$ 3
Insoluble residue	16 $\pm$ 4	9 $\pm$ 2
CO ₂	34 $\pm$ 4	30 $\pm$ 4
Metabolized glucose	355 $\pm$ 36	368 $\pm$ 46
O ₂ uptake (μ moles/g./ 90 min.)	99 $\pm$ 4	76 $\pm$ 5

* Mostly unchanged substrate.

Table 3. Influence of atractyloside on [U-¹⁴C]-glucose metabolism in rat diaphragm muscle incubated for 3 hr. with 2.8 mM-glucose

Experimental conditions were as described in Table 1. The glucose concentration was 2.8 mM and incubation was for 3 hr. Mean values of two experiments $\pm$ s.e.m. are given.

	Glucose converted (μ g./100 mg. of tissue)	
	No atractyloside	With atractyloside
Glycogen and oligo- saccharides	35 $\pm$ 4	15 $\pm$ 2
Zone g*	28 $\pm$ 1	25 $\pm$ 1
Lactate	183 $\pm$ 1	302 $\pm$ 4
Phosphoglycerate	80 $\pm$ 6	10 $\pm$ 1
Insoluble residue	26 $\pm$ 4	4 $\pm$ 1
CO ₂	44 $\pm$ 4	34 $\pm$ 3
Metabolized glucose	396 $\pm$ 10	390 $\pm$ 2
O ₂ uptake (μ moles/g./ 180 min.)	153 $\pm$ 4	89 $\pm$ 4

* Mostly unchanged substrate.

as 1, the specific radioactivity of the lactate was 0.46 and was not influenced by the presence of atractyloside.

In four other experiments, in which total glycogen was isolated by the method of Shaw & Stadie (1957), the amount of radioactive glucose incorporated into glycogen decreased, in the presence of

Table 4. Effect of atractyloside on [U-¹⁴C]pyruvate metabolism in rat diaphragm muscle

Incubation was for 90 min. in 1.2 ml. of phosphate-buffered medium, pH 6.8, at 37° in O₂. The pyruvate concentration was 9.1 mM (approx. 20 μ C/vessel); the atractyloside concentration was 1.2 mM. Mean values of six experiments $\pm$ s.e.m. are given.

	Pyruvate converted (μ g./100 mg. of tissue)	
	No atractyloside	With atractyloside
Glycogen and oligo- saccharides	3 $\pm$ 1	1 $\pm$ 0.1
Lactate	460 $\pm$ 14	465 $\pm$ 16
Alanine	63 $\pm$ 8	64 $\pm$ 7
Glutamate	13 $\pm$ 1	18 $\pm$ 2
Glutamine	6 $\pm$ 1	3 $\pm$ 0.6
CO ₂	190 $\pm$ 33	160 $\pm$ 8
Metabolized pyruvate	735 $\pm$ 33	711 $\pm$ 30
O ₂ uptake (μ moles/g./ 90 min.)	117 $\pm$ 5	88 $\pm$ 4

Fig. 2. Effect of atractyloside on ¹⁴CO₂ production from [1-¹⁴C]pyruvate and [3-¹⁴C]pyruvate at various incubation times in rat diaphragm muscle. Tissue (100 mg.) was incubated in 1.2 ml. of phosphate-buffered medium, pH 6.8, at 37° in O₂. The pyruvate concentration was 9.1 mM and that of atractyloside, when present, was 1.2 mM. ○, [1-¹⁴C]Pyruvate without atractyloside; △, [1-¹⁴C]pyruvate with atractyloside; ●, [3-¹⁴C]pyruvate without atractyloside; ▲, [3-¹⁴C]pyruvate with atractyloside.

the glucoside, from 36 $\pm$ 3 μ g. to 16 $\pm$ 3 μ g., in good agreement with the results shown in Table 2.

Pyruvate metabolism. With pyruvate as substrate the oxygen uptake of diaphragm was also decreased by about 25% by atractyloside. The

Table 5. *Effect of atractyloside on $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{pyruvate}$ in rat diaphragm muscle at various incubation times*

Experimental conditions were as described in Table 4. The pyruvate concentration was 91mM (approx. $1\mu\text{C}/\text{vessel}$). Mean values of two experiments $\pm$ S.E.M. are given.

Incubation time (hr.)	Pyruvate converted ($\mu\text{g.}/100\text{mg. of tissue}$)	
	No atractyloside	With atractyloside
0.5	240 ± 20	200 ± 15
1.0	460 ± 30	390 ± 25
2.0	859 ± 60	671 ± 40
3.0	1203 ± 79	948 ± 61

production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]\text{pyruvate}$ was decreased by about 16%, in good agreement with the diminished oxygen uptake (Table 4). No effect of the glucoside on the incorporation of radioactivity into other metabolites was observed.

With $[3\text{-}^{14}\text{C}]\text{pyruvate}$ the effect on $^{14}\text{CO}_2$ production became rather more evident, but with $[1\text{-}^{14}\text{C}]\text{pyruvate}$ no decrease was noticed over an incubation period of 3 hr. (Fig. 2). With a tenfold increase in pyruvate concentration to 91mM some decrease was noted even with $[1\text{-}^{14}\text{C}]\text{pyruvate}$, varying from 15 to 20% with increased incubation time (Table 5).

To elucidate this point the effect of atractyloside was studied on pyruvate dismutation (Krebs & Johnson, 1937; Weil-Malherbe, 1937) at various concentrations of pyruvate. However, the glucoside did not influence the production of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{pyruvate}$ in anaerobiosis in the concentration range 0.091–91mM and therefore appeared to be without effect on the non-oxidative dismutation of pyruvate.

Calculated specific radioactivity of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{pyruvate}$. The μmoles of $^{14}\text{CO}_2$ produced/ μmole of oxygen consumed with various concentrations of $[1\text{-}^{14}\text{C}]\text{pyruvate}$ after 90 min. of incubation are given in Table 6, which shows that the ratio rises from 3.1×10^{-4} to 5.7×10^{-1} as the concentration of the substrate is varied from 0.091 to 91mM. Considering that the specific radioactivity of the $^{14}\text{CO}_2$ with $[1\text{-}^{14}\text{C}]\text{pyruvate}$ should in no case be higher than 0.40 (the value corresponding to the labelling of all the tricarboxylic acid-cycle intermediates), the finding of a value of 0.57 with 91mM- $[1\text{-}^{14}\text{C}]\text{pyruvate}$ suggests that at this concentration about one-third of the total carbon dioxide is not produced via the tricarboxylic acid cycle.

Atractyloside increased the specific radioactivity

Table 6. *Influence of atractyloside on the calculated specific radioactivity of $^{14}\text{CO}_2$ at various concentrations of $[1\text{-}^{14}\text{C}]\text{pyruvate}$*

Experimental conditions were as described in Table 4. The pyruvate concentrations were as given (approx. $1\mu\text{C}/\text{vessel}$). Calculated specific radioactivity is expressed as μmoles of $^{14}\text{CO}_2$ produced/ μmole of O_2 consumed. Mean values of four experiments $\pm$ S.E.M. are given.

Concn. of pyruvate (mM)	$10^4 \times$ Calc. specific radioactivity	
	No atractyloside	With atractyloside
0.091	3.1 ± 0.1	4.1 ± 0.5
0.91	27.0 ± 2.4	40.0 ± 6.0
9.1	145.0 ± 12.0	193.0 ± 12.0
91.0	5687.0 ± 121.0	5900.0 ± 240.0

of the $^{14}\text{CO}_2$ at the lowest concentrations of pyruvate and had no effect when the substrate concentration was 91mM.

DISCUSSION

The results reported in the present paper show that atractyloside acts on intact tissue *in vitro*. The effect on oxygen uptake and on $^{14}\text{CO}_2$ formation, whether from labelled glucose or from labelled pyruvate, suggests that atractyloside enters the cell and acts at the mitochondrial level.

The results on the production of $^{14}\text{CO}_2$ from pyruvate labelled in different carbon atoms and on the calculated specific radioactivity of the $^{14}\text{CO}_2$ suggest that carbon dioxide produced by the tissue originates partly from the further oxidation of pyruvate and partly from dismutation. The inhibitory role of atractyloside would seem to be the resultant of two components: inhibition of the oxidative production of carbon dioxide and stimulation of carbon dioxide production due to dismutation primed by the consequent rise in pyruvate concentration. This interpretation is supported by the following evidence: (a) with $[3\text{-}^{14}\text{C}]\text{pyruvate}$ the $^{14}\text{CO}_2$ production at any concentration and at any incubation time was decreased in correspondence to the diminished oxygen uptake, whereas with $[1\text{-}^{14}\text{C}]\text{pyruvate}$ at 9.1mM concentration there was no such decrease in the production of $^{14}\text{CO}_2$; (b) atractyloside did not appreciably affect the anaerobic formation of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{pyruvate}$.

A particularly noteworthy point is that atractyloside increased the specific radioactivity of $^{14}\text{CO}_2$ from $[1\text{-}^{14}\text{C}]\text{pyruvate}$ at the lowest substrate concentrations, but had no effect at the higher concentration of 91mM (Table 6). This is probably due to the fact that at high pyruvate concentration

dismutation proceeds maximally and therefore cannot be further stimulated. At lower substrate concentrations the stimulating effect of atractyloside on the specific radioactivity becomes evident, suggesting that the increased intracellular concentration of pyruvate consequent on inhibition of oxidation results in a higher rate of dismutation.

With 5.6 mm-glucose, the concentration used in our experiments, no significant compensation attributable to the dismutation was seen, even when [3,4-¹⁴C₂]glucose was used. This probably indicates that at this concentration of substrate the concentration of pyruvate in the cell is not sufficiently high to bring about dismutation.

The marked increase in lactate formation induced by atractyloside with glucose as substrate is presumably due to stimulation of phosphorylation at the substrate level related to phosphoglycerate. Indeed the fall in ATP concentration and the rise in that of ADP in the cytoplasm, caused by changes in the normal replacement by mitochondrial activity (Bruni & Azzone, 1964; Kemp & Slater, 1964; Chappell & Crofts, 1965; Heldt, Jacobs & Klingenberg, 1965; Bruni *et al.* 1965; Brierley & O'Brien, 1965; Vignais *et al.* 1966; Moret *et al.* 1966), stimulate reactions in the anaerobic catabolism that lead to synthesis of ATP. Thus one sees a decrease in the phosphoglycerate concentration and a rise in that of radioactive lactate.

Finally, the diminished incorporation of radioactivity into glycogen can be attributed to inhibition of synthesis as a result of a deficiency of energy-rich nucleotides.

In summary, the alterations induced by the atractyloside in the metabolism of rat diaphragm suggest that the glucoside gives rise in the tissue to conditions of partial anaerobiosis.

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