Uncoupling Reagents and Metabolism

5. EFFECTS OF SALICYLATE AND OF 2,4-DINITROPHENOL ON THE METABOLISM OF ISOLATED RAT DIAPHRAGM*

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Salicylate and 2,4-dinitrophenol produce effects on the incorporation and distribution of radioactivity from labelled glucose, pyruvate and acetate into the soluble intermediates of chopped preparations of isolated rat tissues (Smith & Moses, 1960; Huggins, Smith & Moses, 1961b). Chopped preparations of tissues inevitably undergo some damage during their preparation and it is difficult to assess with accuracy their degree of structural and metabolic integrity. In the present work similar experiments have been performed with the isolated rat-diaphragm muscle because this tissue is relatively undamaged compared with the chopped preparations, allows other chemical determinations, e.g. of glucose uptake and glycogen content, to be easily made, and may be readily separated from the incubation medium. A preliminary account of part of this work has been published (Huggins & Smith, 1961).

EXPERIMENTAL

Preparation of diaphragm. Female rats (wt. 100–120 g.) of the Wistar strain, maintained on Medical Research Council cube diet no. 41, were starved for 24 hr. before being killed by stunning and cervical fracture. The diaphragm was removed by cutting round the sternal and rib boundaries, care being taken to cause the minimum of damage to the muscle fibres. The excised diaphragm was washed in the appropriate incubation medium for 5 sec. to remove any blood, and transferred to a sintered-glass filter-crucible containing approx. 10 ml. of the incubation medium, which was gassed with either O_2 or $O_2 + CO_2$ (95:5). The diaphragm was divided into two hemidiaphragms and a central portion, the latter being immediately placed on solid CO_2 and subsequently used for glycogen estimation. After 90 sec. the hemidiaphragms were removed separately from the filter, the connective tissue was removed and each was divided into two quarter-diaphragms. These were blotted carefully with Whatman no. 50 filter paper, weighed, and placed in the incubation vessels.

Incubation conditions. Three incubation media were used: Krebs–Ringer phosphate (Umbreit, Burris & Stauffer,

1957), Krebs–Ringer bicarbonate (Umbreit *et al.* 1957) and Gey's bicarbonate (Gey & Gey, 1936). The incubation media were adjusted to pH 7.4 with either 0.01N-HCl or 0.01N-NaOH for the phosphate medium and by gassing with $O_2 + CO_2$ (95:5) at the incubation temperature for the bicarbonate medium. The media contained either 150 mg. of glucose/100 ml. (Krebs–Ringer phosphate and bicarbonate) or 250 mg. of glucose/100 ml. (Gey's bicarbonate).

The diaphragms were incubated for 1 hr. with shaking (at 100 cyc./min.) at 37° in 2 ml. beakers held in air-tight boxes (Huggins, Martin & Ottaway, 1959). Each quarter-diaphragm was incubated in 0.5 ml. of medium and each hemidiaphragm in 1.0 ml. of medium. The boxes were gassed for 5 min. with $O_2 + CO_2$ (95:5) for the bicarbonate medium and 100% O_2 for the phosphate medium. In the radioactivity experiments the uncoupling reagents were made up in the appropriate incubation medium to give a final concentration of 5.0 mM for sodium salicylate and 0.25 mM for 2,4-dinitrophenol, but in some experiments a wider range of 2,4-dinitrophenol concentrations was used. Solutions (0.005 ml. quantities) of the radioactive substrates, [^{14}C]glucose (5 μ C, 0.07 μ mole), sodium [$2-^{14}C$]acetate (5 μ C, 1 μ mole) and sodium [$3-^{14}C$]pyruvate (5 μ C, 1.4 μ moles), obtained from The Radiochemical Centre, Amersham, Bucks., were added before the beginning of each experiment.

At the end of the incubation period the diaphragms were removed; 0.05 ml. samples of the incubation medium were taken without deproteinization for the estimation of glucose with glucose oxidase (Huggett & Nixon, 1957), and 0.2 ml. samples for the determination of lactic acid (Barker & Summerson, 1941). In the radioactivity experiments additional samples (0.1 ml.) of the incubation medium were used for chromatography. The treatment of the diaphragm at the end of the incubation varied with the type of experiment. When radioactive material was present each quarter-diaphragm was removed from the incubation vessel, rinsed rapidly in a beaker of the medium and placed in 2 ml. of boiling 95% (v/v) ethanol. After 30 min. the quarter-diaphragms were removed and extracted with 2 ml. of 70% (v/v) ethanol, followed after a further 30 min. by 1 ml. of 50% (v/v) ethanol. These extracts were combined and concentrated, and samples (25%) were used for chromatography. The methods used for the chromatographic separation and measurement of radioactivity in the soluble intermediates were those described by Smith & Moses (1960). The extracted diaphragms were then dissolved in 1 ml. of boiling 40% (w/v) KOH and analysed for glycogen (Good, Kramer & Somogyi, 1933), the glucose formed on hydrolysis being estimated by glucose oxidase

* Part 4: Smith & Moses (1961).

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(Huggett & Nixon, 1957). In experiments without radioactive substrates the diaphragms were removed, blotted and placed in 1 ml. of 40% (w/v) KOH for glycogen analysis.

RESULTS

Effects of salicylate and of 2,4-dinitrophenol on diaphragm metabolism. The results in Table 1 show that 5 mM-salicylate and 0.25 mM-2,4-dinitrophenol decrease the glycogen content and increase the lactic acid accumulation in the quarter-diaphragms incubated in either medium. Both uncoupling reagents inhibited the glucose uptake in the phosphate medium but only 2,4-dinitrophenol produced this effect in the bicarbonate medium. These results agree with those of Pierce & Field (1949) and Smith & Jeffrey (1956). However, Randle & Smith (1958*a*) reported that both 0.05–0.25 mM-2,4-dinitrophenol and 5 mM-salicylate caused a marked stimulation of the glucose uptake of rat hemidiaphragms incubated in Gey's bicarbonate medium in the presence of 250 mg. of glucose/100 ml. This effect did not occur with the quarter-diaphragms incubated in Krebs–Ringer bicarbonate containing 150 mg. of glucose/100 ml. (Table 1), and unlike Randle & Smith (1958*a*) we

observed no stimulation of glucose uptake with hemidiaphragms incubated in Gey's bicarbonate medium with 250 mg. of glucose/100 ml. in the presence of either 5 mM-salicylate or 0.25 mM-2,4-dinitrophenol (Table 2).

The results in Table 3 show the effects of 2,4-dinitrophenol concentrations, ranging from 0.0005 to 0.5 mM, on the glycogen content, lactic acid accumulation and glucose uptake of quarter-diaphragms incubated in Krebs–Ringer bicarbonate. All the concentrations of 2,4-dinitrophenol, with the exception of 0.0005 mM, caused significant depletions of the glycogen and accumulations of lactic acid; 0.005 mM- and 0.01 mM-2,4-dinitrophenol had no significant effect on the glucose uptake but 0.25 mM- and 0.5 mM-2,4-dinitrophenol significantly decreased the glucose uptake. Sodium cyanide (mM) did not change the glycogen content of the preparation but produced significant increases in the lactic acid accumulation and glucose uptake. The last effect was also observed by Randle & Smith (1958*a*) with hemidiaphragms in Gey's bicarbonate medium.

Incorporation and distribution of ^{14}C from [^{14}C]glucose, [^{14}C]acetate and [^{14}C]pyruvate into the soluble intermediates. The results in Table 4 show

Table 1. *Effects of salicylate and of 2,4-dinitrophenol on the metabolism of rat quarter-diaphragms incubated in either Krebs–Ringer phosphate or Krebs–Ringer bicarbonate media*

Each vessel contained 0.5 ml. of medium and approx. 50 mg. wet wt. of tissue. The final glucose concentration was 1.5 mg./ml. The incubation time was 60 min. at 37° in the presence or absence of salicylate or 2,4-dinitrophenol. The results are expressed as the mean $\pm$ s.e.m., the number of observations being given in parentheses.

Medium	Addition (final concn.)	Glycogen content (mg./g. wet wt.)	Difference $\pm$ s.e. of difference	Significance of difference (P)
Krebs–Ringer phosphate	None	2.39 $\pm$ 0.20 (14)	—	—
	Sodium salicylate (5 mM)	0.41 $\pm$ 0.13 (6)	– 1.98 $\pm$ 0.18	0.02
	2,4-Dinitrophenol (0.25 mM)	0.28 $\pm$ 0.02 (8)	– 2.11 $\pm$ 0.07	0.001
Krebs–Ringer bicarbonate	None	2.47 $\pm$ 0.22 (16)	—	—
	Sodium salicylate (5 mM)	0.18 $\pm$ 0.10 (8)	– 2.29 $\pm$ 0.14	0.001
	2,4-Dinitrophenol (0.25 mM)	0.23 $\pm$ 0.13 (8)	– 2.24 $\pm$ 0.25	0.001
Medium	Addition (final concn.)	Lactic acid accumulation (mg./g. wet wt./hr)	Difference $\pm$ s.e. of difference	Significance of difference (P)
Krebs–Ringer phosphate	None	1.69 $\pm$ 0.13 (14)	—	—
	Sodium salicylate (5 mM)	2.30 $\pm$ 0.15 (6)	+ 0.61 $\pm$ 0.12	0.02
	2,4-Dinitrophenol (0.25 mM)	2.75 $\pm$ 0.34 (8)	+ 1.06 $\pm$ 0.25	0.01
Krebs–Ringer bicarbonate	None	2.14 $\pm$ 0.20 (16)	—	—
	Sodium salicylate (5 mM)	3.65 $\pm$ 0.16 (8)	+ 1.51 $\pm$ 0.17	0.001
	2,4-Dinitrophenol (0.25 mM)	3.33 $\pm$ 0.30 (8)	+ 1.19 $\pm$ 0.29	0.01
Medium	Addition (final concn.)	Glucose uptake (mg./g. wet wt./hr.)	Difference $\pm$ s.e. of difference	Significance of difference (P)
Krebs–Ringer phosphate	None	2.10 $\pm$ 0.17 (14)	—	—
	Sodium salicylate (5 mM)	1.36 $\pm$ 0.17 (6)	– 0.74 $\pm$ 0.14	0.02
	2,4-Dinitrophenol (0.25 mM)	1.07 $\pm$ 0.11 (8)	– 1.03 $\pm$ 0.12	0.001
Krebs–Ringer bicarbonate	None	2.36 $\pm$ 0.13 (16)	—	—
	Sodium salicylate (5 mM)	2.61 $\pm$ 0.12 (8)	+ 0.25 $\pm$ 0.16	0.2
	2,4-Dinitrophenol (0.25 mM)	1.40 $\pm$ 0.19 (8)	– 0.96 $\pm$ 0.20	0.01

Table 2. *Effects of salicylate and of 2,4-dinitrophenol on the glucose uptake of isolated rat hemidiaphragms incubated in Gey's bicarbonate medium*

Each vessel contained 1.0 ml. of medium and approx. 100 mg. wet wt. of tissue. The final glucose concentration was 2.5 mg./ml. The incubation time was 60 min. at 37°. The results are expressed as the mean $\pm$ s.e.m., the number of observations being given in parentheses.

Addition	Glucose uptake (mg./g. wet wt./hr.)	Difference $\pm$ s.e. of difference	Significance of difference (P)
None	2.78 $\pm$ 0.44 (6)	—	—
Sodium salicylate (5 mM)	2.90 $\pm$ 0.40 (6)	+ 0.12 $\pm$ 0.28	0.7
2,4-Dinitrophenol (0.25 mM)	2.03 $\pm$ 0.50 (6)	- 0.73 $\pm$ 0.22	0.05

Table 3. *Effects of 2,4-dinitrophenol and of sodium cyanide on the metabolism of isolated rat quarter-diaphragms incubated in Krebs-Ringer bicarbonate medium*

Each vessel contained 0.5 ml. of medium and approx. 50 mg. wet wt. of tissue. The final glucose concentration was 1.5 mg./ml. The incubation time was 60 min. at 37° in the presence or absence of either 2,4-dinitrophenol or sodium cyanide. The results are expressed as the mean $\pm$ s.e.m., the number of observations being given in parentheses.

Addition (final concn.)	Glycogen content (mg./g. wet wt.)	Difference $\pm$ s.e. of difference	Significance of difference (P)
None	2.59 $\pm$ 0.11 (4)	- 0.21 $\pm$ 0.11	0.2
2,4-Ditrophenol (0.0005 mM)	2.38 $\pm$ 0.14 (4)		
None	2.80 $\pm$ 0.07 (6)	- 0.90 $\pm$ 0.26	0.05
2,4-Dinitrophenol (0.005 mM)	1.90 $\pm$ 0.21 (6)		
None	3.06 $\pm$ 0.17 (8)	- 1.66 $\pm$ 0.15	0.001
2,4-Dinitrophenol (0.01 mM)	1.38 $\pm$ 0.12 (8)		
None	3.08 $\pm$ 0.31 (4)	- 2.08 $\pm$ 0.16	0.01
2,4-Dinitrophenol (0.05 mM)	1.00 $\pm$ 0.18 (4)		
None	2.63 $\pm$ 0.30 (8)	- 2.40 $\pm$ 0.25	0.001
2,4-Dinitrophenol (0.25 mM)	0.23 $\pm$ 0.13 (8)		
None	2.23 $\pm$ 0.05 (4)	- 2.12 $\pm$ 0.24	0.01
2,4-Dinitrophenol (0.5 mM)	0.11 $\pm$ 0.01 (4)		
None	2.32 $\pm$ 0.17	- 0.13 $\pm$ 0.06	0.2
Sodium cyanide (1 mM)	2.19 $\pm$ 0.12		
Addition (final concn.)	Lactic acid accumulation (mg./g. wet wt./hr.)	Difference $\pm$ s.e. of difference	Significance of difference (P)
None	1.89 $\pm$ 0.36 (4)	+ 0.04 $\pm$ 0.13	0.8
2,4-Dinitrophenol (0.0005 mM)	1.93 $\pm$ 0.29 (4)		
None	2.70 $\pm$ 0.31 (6)	+ 1.05 $\pm$ 0.26	0.02
2,4-Dinitrophenol (0.005 mM)	3.75 $\pm$ 0.40 (6)		
None	1.82 $\pm$ 0.18 (8)	+ 1.18 $\pm$ 0.15	0.001
2,4-Dinitrophenol (0.01 mM)	3.00 $\pm$ 0.23 (8)		
None	2.01 $\pm$ 0.28 (4)	+ 1.60 $\pm$ 0.26	0.02
2,4-Dinitrophenol (0.05 mM)	3.61 $\pm$ 0.45 (4)		
None	2.04 $\pm$ 0.24 (8)	+ 1.29 $\pm$ 0.29	0.01
2,4-Dinitrophenol (0.25 mM)	3.33 $\pm$ 0.30 (8)		
None	2.16 $\pm$ 0.32 (4)	+ 0.96 $\pm$ 0.13	0.01
2,4-Dinitrophenol (0.5 mM)	3.12 $\pm$ 0.32 (4)		
None	2.85 $\pm$ 0.16 (8)	+ 1.22 $\pm$ 0.26	0.01
Sodium cyanide (1 mM)	4.07 $\pm$ 0.25 (8)		
Addition (final concn.)	Glucose uptake (mg./g. wet wt./hr.)	Difference $\pm$ s.e. of difference	Significance of difference (P)
None	2.56 $\pm$ 0.11 (4)	- 0.04 $\pm$ 0.16	0.8
2,4-Dinitrophenol (0.0005 mM)	2.52 $\pm$ 0.11 (4)		
None	2.66 $\pm$ 0.12 (6)	+ 0.23 $\pm$ 0.14	0.3
2,4-Dinitrophenol (0.005 mM)	2.89 $\pm$ 0.11 (6)		
None	2.57 $\pm$ 0.09 (8)	+ 0.31 $\pm$ 0.13	0.1
2,4-Dinitrophenol (0.01 mM)	2.88 $\pm$ 0.13 (8)		
None	2.83 $\pm$ 0.12 (4)	- 0.31 $\pm$ 0.19	0.3
2,4-Dinitrophenol (0.05 mM)	2.52 $\pm$ 0.16 (4)		
None	2.55 $\pm$ 0.12 (8)	- 1.05 $\pm$ 0.22	0.01
2,4-Dinitrophenol (0.25 mM)	1.50 $\pm$ 0.18 (8)		
None	2.65 $\pm$ 0.13 (4)	- 1.48 $\pm$ 0.24	0.02
2,4-Dinitrophenol (0.5 mM)	1.17 $\pm$ 0.24 (4)		
None	3.00 $\pm$ 0.12 (8)	+ 1.17 $\pm$ 0.19	0.001
Sodium cyanide (1 mM)	4.17 $\pm$ 0.17 (8)		

the amounts of ¹⁴C from [¹⁴C]glucose incorporated into the soluble intermediates of quarter-diaphragms incubated in either Krebs–Ringer phosphate or Krebs–Ringer bicarbonate in the presence or the absence of salicylate or of 2,4-dinitrophenol. In the absence of the inhibitors the qualitative pattern of incorporation from the labelled glucose was similar in both media. Radioactivity was found mainly in the amino acids, organic acids, maltose and the phosphate fraction. The pattern of distribution of ¹⁴C was similar to that observed with chopped preparations of rat tissues (Smith & Moses, 1960), but differ from those reported by Chain, Beloff-Chain & Pocchiari (1956) and Chain (1959) in that much less ¹⁴C was found in oligosaccharides and also that radioactivity was detected in a greater variety of metabolic intermediates.

Differences were observed between the two media with respect to the quantitative distribution of ¹⁴C in the soluble intermediates. These differences concerned the amounts of radioactivity present in various fractions and the distribution of the ¹⁴C present in the same intermediates between the tissue and the incubation medium. In the phosphate medium approximately 5 times more radioactivity

was incorporated into the soluble fraction, and certain compounds (alanine and aspartic acid) showed a tenfold increase. The increased radioactivity in the soluble fraction in the phosphate medium occurred in the labelled intermediates found in the incubation medium rather than in the same compounds present in the tissue extract. Thus a much greater proportion of the labelled intermediates formed from the [¹⁴C]glucose accumulated in the incubation medium when the quarter-diaphragms were incubated in phosphate medium than when they were incubated in the bicarbonate medium.

The uncoupling reagents caused relatively little change in the distribution of ¹⁴C from the labelled glucose among the soluble intermediates. However, both 2,4-dinitrophenol and salicylate caused substantial increases in the proportions of radioactivity found in the soluble intermediates isolated from the incubation media. These increases occurred in both media and involved the total soluble fraction and in particular the organic acids and amino acids. 2,4-Dinitrophenol, but not salicylate, increased the amount of [¹⁴C]glucose found in the separated tissue at the end of the experiments irrespective of the incubation medium used (Table 5). A similar accumulation of glucose

Table 4. Incorporation and distribution of ¹⁴C-labelled soluble intermediates derived from [¹⁴C]glucose by rat diaphragm incubated in Krebs–Ringer phosphate or Krebs–Ringer bicarbonate in the presence or absence of salicylate or of 2,4-dinitrophenol

Each vessel contained 0.5 ml. of medium and approx. 50 mg. wet wt. of tissue. The final glucose concentration was 1.5 mg./ml. The incubation time was 60 min. at 37°, with 5 μC of [¹⁴C]glucose in the presence or absence of either 5 mM-salicylate or 0.25 mM-2,4-dinitrophenol. The Table shows the radioactivity (expressed as counts/min./mg. wet wt. of tissue) present in each substance in the separated incubation medium and in the tissue. Control results are the means of four observations and experimental results are the means of two observations.

Uncoupling reagent ...	In Krebs–Ringer phosphate						In Krebs–Ringer bicarbonate					
	None (control)		Salicylate		2,4-Dinitrophenol		None (control)		Salicylate		2,4-Dinitrophenol	
	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue
Alanine	651	36	568	37	683	30	22	28	19	20	61	21
Aspartic acid	380	13	492	5	494	18	24	19	29	2	17	5
Glutamic acid	0	15	0	0	0	6	9	6	12	7	12	5
Glutamine	0	7	0	7	0	6	0	0	0	0	0	0
γ-Aminobutyric acid	105	8	155	5	140	7	29	5	25	1	0	0
Citric acid	0	7	0	3	0	3	4	2	5	2	6	1
Lactic acid	320	42	215	24	207	12	166	92	199	44	171	23
Malic acid	19	6	43	1	0	4	1	1	0	0	2	1
Maltose	101	18	114	8	139	16	55	5	78	1	92	2
Oligosaccharides	0	0	0	0	0	0	16	4	22	2	32	0
Phosphate fraction	326	20	284	34	318	20	71	24	46	13	37	5
Unidentified compounds	465	42	426	32	547	28	30	1	44	10	30	0
Total in soluble fraction	2367	214	2297	156	2528	150	427	187	479	102	460	63

Table 5. Effect of 2,4-dinitrophenol and of salicylate on the amounts of [¹⁴C]glucose present in rat quarter-diaphragms in either Krebs–Ringer phosphate or Krebs–Ringer bicarbonate media

Experimental details were as given for Table 4. The results are given as counts/min./mg. wet wt. of tissue.

Addition ...	In Krebs–Ringer phosphate			In Krebs–Ringer bicarbonate		
	None	Salicylate	2,4-Dinitrophenol	None	Salicylate	2,4-Dinitrophenol
Glucose	544	345	1015	300	323	621

in intact rat-diaphragm muscle in the presence of 2,4-dinitrophenol was reported by Randle & Smith (1958b).

Table 6 gives the results of similar experiments with $[2-^{14}\text{C}]$ acetate as the labelled substrate. In the absence of the uncoupling reagents the qualitative pattern of incorporation of the ^{14}C was similar in both media and resembled that observed with chopped preparations of rat tissues (Smith & Moses, 1960). Radioactivity was found mainly in the amino acids alanine, aspartic acid and glutamic acid, in glutamine, and in the organic acids citric acid, malic acid and lactic acid, with smaller amounts in fumaric acid and succinic acid. In the

bicarbonate medium approximately twice as much ^{14}C was incorporated into the total soluble fraction. This increased incorporation of radioactivity was particularly associated with the glutamic acid and the glutamine present in the tissue extract. These results differ from those obtained with the $[^{14}\text{C}]$ -glucose (Table 4) in which the increased radioactivity occurred in the phosphate medium and was mainly concerned with the labelled alanine and aspartic acid found in the separated incubation medium.

Salicylate and 2,4-dinitrophenol decreased the total incorporation of ^{14}C from the labelled acetate into the soluble intermediates in a similar manner

Table 6. *Incorporation and distribution of ^{14}C -labelled soluble intermediates derived from $[2-^{14}\text{C}]$ acetate by rat diaphragm incubated in Krebs-Ringer phosphate or Krebs-Ringer bicarbonate in the presence or absence of salicylate or of 2,4-dinitrophenol*

Experimental details were as given for Table 4 except that $5\text{ }\mu\text{C}$ of sodium $[2-^{14}\text{C}]$ acetate was used.

Uncoupling agent	In Krebs-Ringer phosphate						In Krebs-Ringer bicarbonate					
	None (control)		Salicylate		2,4-Dinitrophenol		None (control)		Salicylate		2,4-Dinitrophenol	
	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue
Alanine	33	7	0	0	0	0.3	19	39	7	3	4	10
Aspartic acid	34	26	24	11	12	10	28	61	23	9	13	13
Glutamic acid	86	305	58	143	53	13	214	562	60	151	19	25
Glutamine	264	276	33	41	35	2	607	1313	90	154	4	144
Proline	20	20	29	7	23	0	0	0	0	0	0	0
Citric acid	22	167	9	37	10	3	124	239	10	6	9	1
Fumaric acid	22	24	0	8	0	0	15	72	15	5	6	1
Lactic acid	341	183	190	35	91	39	240	157	105	42	214	11
Malic acid	187	122	62	12	40	3	123	151	75	23	41	1
Succinic acid	21	48	27	0	0	0	10	120	62	16	7	1
Phosphate fraction	0	2	0	0	0	0	45	7	12	2	10	0.5
Unidentified compounds	60	120	13	17	0	0	135	181	23	35	9	2
Total in soluble fraction	1040	1300	445	311	264	70	1560	2902	482	446	336	210

Table 7. *Incorporation and distribution of ^{14}C -labelled soluble intermediates derived from $[3-^{14}\text{C}]$ pyruvate by rat diaphragm incubated in Krebs-Ringer phosphate or Krebs-Ringer bicarbonate in the presence or absence of salicylate or of 2,4-dinitrophenol*

Experimental details were as given for Table 4 except that $5\text{ }\mu\text{C}$ of sodium $[3-^{14}\text{C}]$ pyruvate was used.

Uncoupling reagent	In Krebs-Ringer phosphate						In Krebs-Ringer bicarbonate					
	None (control)		Salicylate		2,4-Dinitrophenol		None (control)		Salicylate		2,4-Dinitrophenol	
	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue
Alanine	651	290	360	97	781	140	80	35	62	10	95	15
Aspartic acid	52	9	68	9	55	12	14	3	11	2	14	3
Glutamic acid	483	68	604	63	530	46	365	50	507	40	480	31
Glutamine	87	109	147	100	14	1	34	93	31	45	0	0
Citric acid	55	2	56	1	116	1	0	7	0	2	0	0
Fumaric acid	35	7	50	8	33	1	11	1	11	1	9	1
Lactic acid	2072	273	1334	155	1709	165	211	20	201	14	143	16
Malic acid	34	7	49	10	63	1	0	6	0	3	0	0
Succinic acid	0	0	47	0	0	3	0	14	0	5	0	4
Glucose	0	0	0	0	0	0	12	0	5	0	5	0
Phosphate fraction	111	1	19	1	51	1	0	4	0	1	0	0
Unidentified compounds	28	72	0	31	26	9	0	10	0	0	0	3
Total in soluble fraction	3608	887	2734	475	3383	380	727	243	828	123	746	73

to that reported for chopped preparations of rat tissues (Smith & Moses, 1960). More radioactivity was found in glutamic acid in both media in the presence of salicylate than when 2,4-dinitrophenol was present, but both reagents reduced the incorporation of ^{14}C into the glutamine in either the phosphate or the bicarbonate medium.

The results in Table 7 are derived from similar experiments with $[3\text{-}^{14}\text{C}]\text{pyruvate}$. There was little difference between the two media with respect to the qualitative distribution of ^{14}C among the soluble intermediates which resembled that found for chopped preparations of isolated rat tissues (Huggins *et al.* 1961*b*). Most of the radioactivity was present in alanine, glutamic acid and lactic acid, with smaller amounts in aspartic acid, glutamine, citric acid, malic acid, fumaric acid and the phosphate fraction. Approximately four times as much ^{14}C was incorporated into the soluble intermediates in the experiments with the phosphate medium compared with the corresponding experiments with the bicarbonate medium. With the exception of glutamine, most of the radioactivity which was present in the labelled intermediates was found in the separated incubation medium. There was little difference between the two media with respect to the distribution of radioactivity present in the same intermediates between the tissue extract and incubation medium. The main effects of the uncoupling reagents were to increase the proportion of the labelled intermediates found in the separated incubation medium and to decrease the incorporation of ^{14}C into the phosphate fraction formed in the experiments with the phosphate medium. Salicylate, but not 2,4-dinitrophenol, reduced the formation of labelled alanine, whereas 2,4-dinitrophenol, but not salicylate, almost completely prevented the incorporation of ^{14}C into glutamine in both incubation media.

DISCUSSION

The results show that rat quarter-diaphragms incorporate radioactivity from $[^{14}\text{C}]\text{glucose}$, $[2\text{-}^{14}\text{C}]\text{acetate}$ and $[3\text{-}^{14}\text{C}]\text{pyruvate}$ into substances known to be involved in glycolysis, the tricarboxylic acid cycle, transamination and synthetic reactions. The qualitative patterns of incorporation of ^{14}C from the labelled substrates among the soluble intermediates were similar to those found for chopped preparations of other rat tissues (Smith & Moses, 1960; Huggins *et al.* 1961*b*).

Two incubation media, Krebs-Ringer bicarbonate and Krebs-Ringer phosphate, were used in the present work because Manchester, Randle & Smith (1958) and Randle & Smith (1958*a*) found that uncoupling reagents produced different effects on the glucose uptake of rat-diaphragm preparations

depending on whether bicarbonate-buffered or phosphate-buffered media were employed. They indicated that more significance should be attached to the results with bicarbonate media because this more closely resembled the ionic composition of extracellular fluid, that phosphate media on account of poor buffering power were unsuitable for the incubation of tissues, such as diaphragm muscle, which exhibit a high rate of glycolysis, and that the phosphate ion in high concentration in a bicarbonate-buffered medium is itself capable of influencing glucose uptake. The present results show that the choice of incubation media influences some but not all the metabolic parameters of isolated rat-diaphragm muscle. The accumulation of lactic acid was increased in the bicarbonate medium, in agreement with the results reported by Shaw & Stadie (1959), but there were no significant differences between the two media with respect to the glycogen content, basal glucose uptake (Table 1) or the qualitative distribution of ^{14}C from the labelled substrates among the soluble intermediates of the rat quarter-diaphragms (Tables 4, 6 and 7). However, differences were observed with respect to the quantitative distribution of the radioactivity from each of the labelled substrates among the soluble intermediates. With $[^{14}\text{C}]\text{glucose}$ and $[3\text{-}^{14}\text{C}]\text{pyruvate}$ more radioactivity was incorporated into the soluble intermediates in the phosphate medium than in the bicarbonate medium, whereas the reverse occurred with $[2\text{-}^{14}\text{C}]\text{acetate}$. The increased incorporations of ^{14}C were associated with the following labelled intermediates: alanine and aspartic acid from $[^{14}\text{C}]\text{glucose}$; glutamic acid and glutamine from $[2\text{-}^{14}\text{C}]\text{acetate}$; alanine and lactic acid from $[3\text{-}^{14}\text{C}]\text{pyruvate}$.

When an increased incorporation of radioactivity occurred in a particular substance, the distribution of this labelled intermediate between the tissue extract and the separated incubation medium varied both with the incubation medium and the labelled substrate. The results are summarized in Table 8, in which the distribution of each labelled soluble intermediate between the tissue extract and the incubation medium is expressed as a medium:tissue ratio. Thus with $[^{14}\text{C}]\text{glucose}$ and $[3\text{-}^{14}\text{C}]\text{pyruvate}$ the increased ^{14}C in the soluble fraction was found in the radioactive substances present in the separated phosphate medium rather than in the tissue extract. In contrast the increased incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{acetate}$ which occurred in the labelled intermediates in the bicarbonate medium was associated with those present in the tissue extract. The most prominent examples were the labelled alanine and aspartic acid formed from $[^{14}\text{C}]\text{glucose}$ in the phosphate medium, where over 90 % of these labelled amino acids were found in the separated medium, and the

Table 8. *Distribution of ^{14}C from $[^{14}\text{C}]$ glucose, $[2\text{-}^{14}\text{C}]$ acetate and $[3\text{-}^{14}\text{C}]$ pyruvate in the soluble intermediates of rat quarter-diaphragm between the tissue and the incubation medium*

Experimental details were as given for Table 4. Results are expressed as the medium:tissue ratios, which equal the amount of radioactivity present in each intermediate in the medium divided by the amount of radioactivity present in the same intermediate in the tissue. Sal., Salicylate; Dnp, 2,4-dinitrophenol.

Uncoupling reagent ...	In Krebs-Ringer phosphate								
	$[^{14}\text{C}]$ Glucose			$[2\text{-}^{14}\text{C}]$ Acetate			$[3\text{-}^{14}\text{C}]$ Pyruvate		
	None	Sal.	Dnp	None	Sal.	Dnp	None	Sal.	Dnp
Alanine	18	15	23	5	—	<0.1	2	4	6
Aspartic acid	29	10	27	1	2	1	6	8	5
Glutamic acid	<0.1	—	0.1	0.3	0.4	4	7	10	12
Glutamine	0.1	0.1	0.1	1	0.8	18	1	1	14
Citric acid	0.1	0.3	0.3	0.1	0.3	3	27	56	116
Lactic acid	8	9	17	2	5	2	8	9	10
Maltose	6	14	9	—	—	—	—	—	—
Total in amino acids	14	22	20	0.7	0.7	5	3	4	7
Total in organic acids	6	9	11	1	3	3	8	9	11
Total in soluble fraction	11	15	17	0.8	1	4	4	6	9

Uncoupling reagent ...	In Krebs-Ringer bicarbonate								
	$[^{14}\text{C}]$ Glucose			$[2\text{-}^{14}\text{C}]$ Acetate			$[3\text{-}^{14}\text{C}]$ Pyruvate		
	None	Sal.	Dnp	None	Sal.	Dnp	None	Sal.	Dnp
Alanine	0.8	1	3	0.5	2	0.4	2	6	6
Aspartic acid	1	15	3	0.5	3	1	5	6	5
Glutamic acid	2	2	2	0.4	0.4	0.8	7	13	16
Glutamine	—	—	—	0.5	0.6	<0.1	0.3	0.7	—
Citric acid	2	3	6	1	2	9	<0.1	<0.1	—
Lactic acid	2	5	7	2	3	20	11	14	9
Maltose	11	78	46	—	—	—	—	—	—
Total in amino acids	1	3	3	0.5	0.6	0.2	3	6	12
Total in organic acids	2	4	7	0.7	3	18	5	8	7
Total in soluble fraction	2	5	7	0.5	1	2	3	7	10

radioactive glutamine formed from $[2\text{-}^{14}\text{C}]$ acetate in the bicarbonate medium, where over 60% of the $[^{14}\text{C}]$ glutamine was recovered from the tissue extract. The general conclusion from the results in Table 8 is that a greater proportion of the radioactive soluble intermediates diffused into the incubation medium when the phosphate-buffered medium was used. One possible explanation of this effect is that it is related to the different amounts of each radioactive intermediate formed from each labelled substrate. Increased incorporations of radioactivity from the $[^{14}\text{C}]$ glucose and $[3\text{-}^{14}\text{C}]$ pyruvate were observed in the experiments with the phosphate medium. However, with $[2\text{-}^{14}\text{C}]$ acetate the increased incorporation of ^{14}C occurred in the bicarbonate medium but the medium:tissue ratios were raised in the phosphate medium.

Salicylate (5 mM) and 0.25 mM-2,4-dinitrophenol produced a significant depletion of the glycogen content and a significant accumulation of lactic acid in the diaphragm, irrespective of the incubation medium employed. Both uncoupling reagents significantly reduced the glucose uptake of the

diaphragm preparations incubated in the phosphate medium, and 2,4-dinitrophenol (0.25 and 0.5 mM) produced a similar effect in the bicarbonate medium. Salicylate (5 mM) did not significantly change the glucose uptake of either quarter-diaphragms incubated in Krebs-Ringer bicarbonate containing 150 mg. of glucose/100 ml. or of hemidiaphragms in Gey's bicarbonate containing 250 mg. of glucose/100 ml. Horiach (1959) stated that 2,4-dinitrophenol increased the glucose uptake of rat-diaphragm preparations, and other workers (Manchester *et al.* 1958; Randle & Smith, 1958*a*) reported that 0.25 mM-2,4-dinitrophenol and 5 mM-salicylate caused a marked stimulation of glucose uptake by rat-diaphragm preparations under apparently identical experimental conditions. Seltzer (1962) claimed that 2–4 mM-salicylate produced a similar effect, but Segal, Blair & Weinberg (1960) found that 2 mM-salicylate did not alter the glucose uptake of hemidiaphragms taken from either normal or diabetic rats. We are unable to account for the discrepancies between the reported results. However, the diaphragm prepara-

tions used in the present work showed a similar qualitative response to incubation with mm-sodium cyanide (Table 3) as that reported by Randle & Smith (1958*a*) to occur with their hemidiaphragms. In addition 2,4-dinitrophenol, but not salicylate, increased the amounts of radioactive glucose found in the separated tissue at the end of the incubation (Table 5), and a similar effect of 2,4-dinitrophenol on the glucose space of the isolated diaphragm was recorded by Randle & Smith (1958*b*).

Salicylate and 2,4-dinitrophenol had little effect on the total incorporation of ^{14}C from either the [^{14}C]glucose or the [$3\text{-}^{14}\text{C}$]pyruvate into the soluble intermediates of the diaphragm, but produced a substantial decrease in that from the labelled acetate. These effects were similar to that observed with chopped preparations of other rat tissues (Smith & Moses, 1960) and may be explicable in terms of the entry of the acetate carbon atoms into the metabolic sequences being more dependent on the supply of ATP and hence more sensitive to the action of uncoupling reagents than is the entry of either the glucose or the pyruvate carbon atoms. Further actions of salicylate and 2,4-dinitrophenol which may be related to their uncoupling actions are the reduction in the incorporation of radioactivity in the phosphate fraction from the labelled pyruvate in Krebs-Ringer phosphate and the decreased formation of the [^{14}C]glutamine in the experiments with the labelled acetate. A similar effect on glutamine synthesis has been reported by Messer (1958), who found that both 2,4-dinitrophenol and salicylate inhibit glutamine formation in guinea-pig-brain preparations at concentrations which stimulate the oxygen consumption. Salicylate, but not 2,4-dinitrophenol, reduced the formation of labelled alanine and increased the incorporation of ^{14}C into glutamic acid in the experiments with [$3\text{-}^{14}\text{C}$]pyruvate. This effect is probably related to the inhibition of glutamate-pyruvate-transaminase activity in rat tissues by salicylate but not by 2,4-dinitrophenol (Huggins, Bryant & Smith, 1961*a*). An effect common to both uncoupling reagents was the increase in medium:tissue ratios for each labelled substrate. Thus a greater proportion of the radioactive soluble intermediates diffused into the incubation media in the presence of either salicylate or 2,4-dinitrophenol, which implies that these reagents increased the permeability of the rat-diaphragm preparations.

Hunter & Ford (1955) have reported that inorganic phosphate, at concentrations similar to that of Krebs-Ringer phosphate, inhibits oxidative-phosphorylation reactions in rat-liver mitochondria. It is possible that the increased medium:tissue ratios found when Krebs-Ringer phosphate

was used as the incubation medium rather than when Krebs-Ringer bicarbonate was employed may be related to the uncoupling action of the inorganic phosphate.

SUMMARY

1. The effects of 5 mm-salicylate and 0.25 mm-2,4-dinitrophenol on the incorporation of ^{14}C from [^{14}C]glucose, [$2\text{-}^{14}\text{C}$]acetate and [$3\text{-}^{14}\text{C}$]pyruvate into the soluble intermediates of isolated rat-diaphragm muscle have been studied.

2. In the absence of the uncoupling reagents, the tissue preparations incorporated ^{14}C into substances known to be involved in glycolysis, in transamination, in the tricarboxylic acid cycle and in synthetic reactions.

3. When Krebs-Ringer phosphate was used as the incubation medium a greater proportion of radioactive intermediates was found in the separated incubation medium than when Krebs-Ringer bicarbonate was employed.

4. The effects of salicylate and of 2,4-dinitrophenol on the distribution of ^{14}C among the soluble intermediates were explicable in terms of their known actions on oxidative phosphorylation and on transamination.

5. Salicylate and 2,4-dinitrophenol caused an increased loss of the labelled soluble intermediates from the tissue into the incubation media.

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Some Non-Reducing Carbohydrates in Animal Tissues and Fluids

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The presence of sorbitol in ram seminal vesicles and sheep foetal blood was reported by Hers (1957a, b, 1960a, b), and evidence was also obtained that sorbitol is an intermediate in the conversion of glucose into fructose, the pathway being

The present investigation confirms the presence of sorbitol, and a preliminary account has been given (Britton, 1958, 1960).

METHODS

Deproteinization and desalting. Samples were deproteinized with ZnSO_4 and Ba(OH)_2 (Somogyi, 1945), a 1:10 dilution being used, or with 5% trichloroacetic acid, which was extracted with ether before desalting. Desalting before chromatography was effected with Bio-Deminrolit (The Permutit Co. Ltd., London, W. 4). When it was not desired to remove the reducing sugars the resin was saturated with CO_2 (Woolf, 1953) and made into a column (vol. 2 ml., length 25 cm.). A portion (2–5 ml.) of the deproteinized solution was run slowly through the column, which was then washed with 3–4 ml. of water. Recoveries of glucose, fructose and sorbitol were quantitative. To remove reducing sugars fresh CO_2 -free Bio-Deminrolit was used and 2–5 ml. of the deproteinized solution run slowly through the column in about 1 hr. The column was then washed with CO_2 -free water to give a total volume of eluate of 20 ml. With this technique all but traces of reducing sugar (less than 5% of glucose and fructose appeared in the eluate when 100–1000 μg . of the sugars was put through the column) were removed, but the recovery of glycerol and sorbitol was quantitative. Samples of glycerol, mannitol,

sorbitol and dulcitol remained chromatographically and electrophoretically homogeneous after this treatment. All columns were well washed before use: this was especially necessary to remove formaldehydogenic material.

Chromatography. Paper chromatography was carried out with the solvent systems A, B, C and D of Table 1 on Whatman no. 1 paper. The solutions to be chromatographed were concentrated in a vacuum desiccator and volumes of 20–100 μl . were dried at the origin in a cold air draught. The amounts of extract applied were equivalent to between 1 and 7.5 ml. of the deproteinized filtrate. Non-reducing and reducing carbohydrates were localized by the method of Trevelyan, Procter & Harrison (1950) as modified by Smith (1960) for systems A, B and C. For system D, the method of Britton (1959a) was employed, 40% HF-acetone (1:40, v/v) proving suitable. Periodate-benzidine (Cifonelli & Smith, 1954) was also used to detect non-reducing carbohydrates in the presence of borate but it was much less sensitive and gave no indication of the amount of material on the spot. For reducing carbohydrates aniline-diphenylamine and naphtharesorcinol were used (Smith, 1960).

Elution from the paper after use of solvents A, B and C was effected by conventional techniques with water as the eluting agent. For system D a modified method was employed to remove the boric acid present. The area to be eluted (10–25 cm^2) was cut out and dipped into a solution of 40% HF-acetone (1:20, v/v). The paper was allowed to dry at room temperature for about 1 hr. and was then eluted in the conventional way, contact with glass being avoided. To the eluate (1–2 ml.) was added an approximately equal volume of aq. 0.01% HF and the solution was run slowly through a column of Bio-Deminrolit saturated with CO_2 (vol. 10 ml., length 25 cm.). The column was further washed with 0.01% HF to give a total volume of eluate of 12 ml., the whole procedure taking about 1 hr. This removed boric acid but gave a quantitative recovery of glucose, fructose and sorbitol. The method appears to be generally applicable, but might not be suitable for highly acid-labile substances owing to the formation of fluoroboric acids on the paper.