Uncoupling Reagents and Metabolism

3. EFFECTS OF SALICYLATE AND 2:4-DINITROPHENOL ON THE INCORPORATION OF ^{14}C FROM LABELLED PYRUVATE INTO THE SOLUBLE INTERMEDIATES OF ISOLATED RAT TISSUES*

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Salicylate and 2:4-dinitrophenol produce effects on the incorporation of radioactivity from labelled glucose and acetate into the soluble metabolic intermediates of isolated rat tissues which are explicable in terms of an uncoupling action on oxidative-phosphorylation processes (Smith & Moses, 1960). No qualitative differences between the actions of the two substances were detected. In the present work, [^{14}C]pyruvate being used as a labelled substrate, salicylate, but not 2:4-dinitrophenol, was found to increase the incorporation of ^{14}C into glutamic acid in isolated preparations of rat liver and kidney. Glutamic-pyruvic-transaminase activity in rat serum was inhibited *in vitro* by salicylate but was not affected by 2:4-dinitrophenol. A preliminary account of part of this work has been published (Huggins, Moses & Smith, 1960).

EXPERIMENTAL

The methods used for the preparation of the tissues (liver, kidney, brain and heart muscle), for the incubation with the labelled substrates in the presence or the absence of either 5 mM-salicylate or 0.5 mM-2:4-dinitrophenol (DNP) and for the chromatographic separation and measurement of radioactivity in the soluble intermediates were those described by Smith & Moses (1960). Sodium [^{14}C]pyruvate (3.62 $\mu\text{C}/\mu\text{mole}$) and sodium L-[^{14}C]glutamate (3.83 $\mu\text{C}/\mu\text{mole}$) were obtained from The Radiochemical Centre, Amersham, Bucks. The labelled glutamate was purified before use by two-dimensional paper chromatography with phenol-water (72%, w/w) as the first solvent and butan-1-ol-propionic acid-water (45.9:23.4:30.6, by vol.) as the second. Approximately 2-3% of the purified material decomposed during the experiments but, with the exception of γ -aminobutyric acid, which formed less than 10% of the decomposition products, the radioactive compounds formed were not metabolized by the kidney preparation used and have not been included in the results.

Glutamic-pyruvic-transaminase and glutamic-oxaloacetic-transaminase activities were measured in the presence

or the absence of either 5 mM-salicylate or 0.5 mM-DNP, by the method of Reitman & Frankel (1957), in pooled serum obtained from adult rats of the Wistar strain.

RESULTS

Total incorporation and distribution of ^{14}C from [^{14}C]pyruvate in the soluble intermediates. The radioactivity measured was that present in the soluble intermediates. The ^{14}C incorporated into substances such as glycogen or proteins, which were insoluble in aqueous ethanol, or that evolved as CO_2 , was not estimated.

The total carbon from the labelled pyruvate which was incorporated into the soluble intermediates of liver, kidney, brain and heart muscle in the presence or the absence of either 5 mM-salicylate or 0.5 mM-DNP is given in Table 1. Brain and kidney were the most active tissues and both uncoupling reagents reduced the incorporation of pyruvate carbon atoms in all the four tissues by about 20-40%. The effect of salicylate was more

Table 1. *Incorporation of ^{14}C from [^{14}C]pyruvate into the soluble intermediates of rat tissues in the presence or the absence of either 5 mM-salicylate or 0.5 mM-2:4-dinitrophenol*

Tissues were cut into blocks and incubated aerobically for 3 hr. at 37° with [^{14}C]pyruvate of known specific activity. Tissues were killed and extracted with ethanol, the extracts were chromatographed and the radioactivity was measured in the separate metabolites. From the quantity of ^{14}C present in the metabolites the total utilization of pyruvate was calculated, and is expressed below as μmoles of pyruvate incorporated into the soluble fraction of 25 mg. of dry tissue in 3 hr. Values are calculated from the sum of the ^{14}C present in the metabolites, in the cell extract and in the medium.

Tissue	Pyruvate incorporation (μmoles)		
	Control	With salicylate	With DNP
Liver	1.4	1.1	1.0
Kidney	2.3	1.5	1.6
Brain	3.4	2.6	2.0
Heart muscle	1.2	1.0	1.1

* Part 2: Moses & Smith (1960).

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pronounced than that of DNP with kidney and heart muscle, and less with liver and brain.

The results in Table 2 show the amounts of ^{14}C incorporated into the soluble intermediates of the four tissues in the presence or the absence of salicylate and DNP.

In the absence of the inhibitors the qualitative pattern of incorporation from the labelled pyruvate was similar for all the tissues. Radioactivity was found in compounds, such as alanine and lactic acid, which were derived from pyruvate itself by transamination and dehydrogenation reactions.

Table 2. *Metabolism of [3- ^{14}C]pyruvate by isolated rat tissues in the presence or the absence of salicylate and 2:4-dinitrophenol*

Each flask contained 1 ml. of tissue suspension (20 mg. dry wt. of liver, 11.1 mg. dry wt. of kidney, 11.5 mg. dry wt. of brain or 10.0 mg. dry wt. of heart muscle) and was incubated with $10\ \mu\text{C}$ of the labelled pyruvate for 3 hr. at 37° in the presence or the absence of either 5 mM-salicylate or 0.5 mM-DNP. Radioactivity is expressed as 10^{-3} counts/min./25 mg. dry wt. of tissue and is the sum of the ^{14}C present in the cell extract and the medium.

Tissue ...	Liver			Kidney		
	None	Salicylate	DNP	None	Salicylate	DNP
Uncoupling reagent ...						
Alanine	323	260	219	300	140	150
γ -Aminobutyric acid	0	0	0	64	4.3	25
Aspartic acid	19	20	25	100	79	52
Glutamic acid	36	69	37	260	320	220
Glutamine	0	0	0	0	0	0
Leucine or isoleucine*	0	0	0	43	28	34
Methionine†	6.4	14	11	24	0	0
Threonine	2.9	3.4	4.6	0	0	0
Glucose‡	34	25	0	67	24	8.1
α -Oxoglutaric acid	0	0	0	0	0	0
Citric acid	21	16	2.1	6.8	27	24
Fumaric acid	4.9	0	6.8	15	14	25
Glyceric acid	0	0	0	85	40	51
Lactic acid	200	92	130	136	57	150
Malic acid	16	27	22	0.4	0	46
Succinic acid	3.4	9	13	13	0	0
Total in						
Amino acids	390	370	300	790	570	480
Organic acids	250	140	170	260	140	300
Glucose	34	25	0	67	24	8.1
Unidentified compounds	5.4	9.7	4.7	26	25	29
Soluble fraction	680	540	470	1100	760	820
Tissue ...	Brain			Heart muscle		
	None	Salicylate	DNP	None	Salicylate	DNP
Uncoupling reagent ...						
Alanine	140	87	130	120	3.5	4.7
γ -Aminobutyric acid	130	14	47	0	0	0
Aspartic acid	270	200	51	16	92	110
Glutamic acid	730	570	220	130	83	130
Glutamine	3.6	2.3	2.3	0	0	0
Leucine or isoleucine*	44	46	92	99	62	66
Methionine†	16	11	1.3	34	17	14
Threonine	12	0	2.5	0	0	0
Glucose‡	25	0	0	0	0	0
α -Oxoglutaric acid	46	0	0	0	0	0
Citric acid	71	100	97	4.6	0	3.1
Fumaric acid	0	0	0	0	0	0
Glyceric acid	76	88	120	73	38	100
Lactic acid	42	21	37	36	33	10
Malic acid	23	14	27	2.1	1.1	4.2
Succinic acid	20	82	40	13	17	5.1
Total in						
Amino acids	1300	930	550	410	260	320
Organic acids	280	300	320	130	90	120
Glucose	25	0	0	0	0	0
Unidentified compounds	70	86	95	80	72	130
Soluble fraction	1700	1300	960	620	420	570

* Contains trace of phenylalanine.

† Contains trace of valine.

‡ Contains trace of serine or glycine or both.

Table 3. *Percentage distribution of ^{14}C from $[3-^{14}\text{C}]$ pyruvate in the soluble intermediates of isolated rat tissues in the presence or the absence of salicylate or 2:4-dinitrophenol*

Details were as given for Table 2. Results are expressed as % of the total ^{14}C present in the sum of all the soluble intermediates.

Tissue ...	Liver			Kidney		
	None	Salicylate	DNP	None	Salicylate	DNP
Uncoupling reagent ...						
Amino acids	57	69	64	72	75	59
Glutamic	5	13	8	24	42	27
Alanine	48	48	47	27	18	18
Aspartic	3	4	5	9	10	6
Organic acids	37	26	36	24	18	37
Lactic acid	29	17	28	12	8	18
Tissue ...	Brain			Heart muscle		
	None	Salicylate	DNP	None	Salicylate	DNP
Uncoupling reagent						
Amino acids	76	72	57	66	62	56
Glutamic	43	44	23	21	20	23
Alanine	8	7	14	19	1	1
Aspartic	16	15	5	3	22	19
Organic acids	16	23	33	21	21	21
Lactic acid	2	2	4	6	8	2

Table 4. *Effects of 5 mM-salicylate and 0.5 mM-2:4-dinitrophenol on transaminase activities in rat serum*

Results are expressed as mean percentage inhibitions ($\pm$ S.E.M.) of the values found in the absence of salicylate or DNP. The results have been analysed by the *t* test, Bessel's correction for small samples being used, and values of *P* are included. The number of observations are given in parentheses.

Transaminase activity	Salicylate	DNP
Glutamic-pyruvic	40 ± 1.4 (8) $P < 0.01$	0.6 ± 0.5 (6) $P > 0.9$
Glutamic-oxaloacetic	11 ± 0.4 (8) $P < 0.05$	0.4 ± 0.8 (6) $P > 0.9$

The occurrence of ^{14}C in citric acid, succinic acid, malic acid and fumaric acid (and in α -oxoglutaric acid in brain) is evidence that the pyruvate carbon atoms entered the tricarboxylic acid cycle. A considerable proportion of the radioactivity was found in amino acids (glutamic and aspartic) which are formed from α -oxoglutarate and oxaloacetate by transamination reactions. The presence of the isotope in methionine and leucine demonstrates the ability of the isolated rat tissues to synthesize amino acids which are generally considered to be essential constituents in the diet. The incorporation of radioactivity into glucose in liver, brain and kidney indicates the presence of synthetic pathways and these tissues also incorporated trace amounts of ^{14}C into substances which were presumptively identified from their chromatographic positions as phosphoenolpyruvate and phosphoglycerate.

In liver, kidney and brain both uncoupling reagents decreased the incorporation of ^{14}C into

glucose; radioactivity, when present, was virtually restricted to the amino and organic acids. A difference was observed between the effects of salicylate and DNP on the distribution of ^{14}C between the amino and organic acid fractions. Salicylate produced proportionately more incorporation of radioactivity into the amino acids than into the organic acids whereas DNP caused the reverse effect (Table 3). Glutamic acid and lactic acid were the substances principally involved in these redistributions and in liver and kidney salicylate caused a greater percentage incorporation of ^{14}C into glutamic acid than occurred in the corresponding control experiments. This effect on glutamic acid was not found with heart muscle, in which both uncoupling reagents produced similar effects in decreasing the accumulation of radioactivity in alanine and increasing it in aspartic acid.

Effects of salicylate and 2:4-dinitrophenol on rat serum transaminase activities. The results in Table 4 show that 5 mM-salicylate caused a significant inhibition *in vitro* of the serum glutamic-pyruvic-transaminase activity and a smaller, but still significant, effect on the serum glutamic-oxaloacetic transaminase activity; 0.5 mM-DNP had no effect on either enzyme activity.

Incorporation of ^{14}C from L- $[^{14}\text{C}]$ glutamate into the soluble intermediates of isolated rat kidney. The results in Table 5 show the amounts of ^{14}C incorporated into the soluble intermediates of kidney in the presence or the absence of salicylate and DNP.

In the absence of the uncoupling reagents radioactivity was found in γ -aminobutyric acid, showing that decarboxylation of the glutamic acid had occurred. The presence of ^{14}C in fumaric acid and

Table 5. *Metabolism of L-[¹⁴C]glutamic acid by rat kidney in the presence or the absence of 5 mM-salicylate or 0.5 mM-2:4-dinitrophenol*

Details were as given for Table 3 except that each flask contained 8.5 mg. dry wt. of kidney, and 4.5 μ C (2.6 μ moles) of labelled glutamic acid was used as substrate.

Uncoupling reagent ...	None	Salicylate	DNP
Glucose	32	0	0
Serine	14	0	0
γ -Aminobutyric acid	67	85	34
Aspartic acid	210	125	173
Fumaric acid	3	14	10
Malic acid	37	49	80
Lactic acid	19	44	53
Unidentified compounds	35	67	45
Total in soluble fraction	415	390	395
Residual glutamic acid	4900	5300	5300

malic acid is evidence that the glutamate carbon atoms entered the tricarboxylic acid cycle and the radioactive aspartic acid was presumably derived by transamination from oxaloacetate. The incorporation of the isotope into lactic acid, serine and glucose indicated the presence of synthetic pathways. The main effects of salicylate and DNP were to prevent the appearance of radiocarbon in serine and glucose and to increase the incorporation of the isotope into the organic acids. DNP caused a decreased appearance of ¹⁴C in γ -aminobutyric acid whereas salicylate had the reverse effect. Both substances had little effect either on the total incorporation of ¹⁴C into the soluble intermediates or on the amount of radioactivity recovered in the residual glutamic acid.

DISCUSSION

Chopped preparations of rat tissues incorporate radioactivity from [^{3-¹⁴C]pyruvate into alanine and lactic acid which are derived directly from the pyruvate by transamination and dehydrogenation reactions. The occurrence of ¹⁴C in citric acid, succinic acid, malic acid and fumaric acid shows that the pyruvate carbon atoms entered the tricarboxylic acid cycle, and a substantial proportion of the radioactivity was found in glutamic acid and aspartic acid, which are formed from α -oxoglutaric acid and oxaloacetic acid by transamination reactions. The presence of synthetic pathways is indicated by the incorporation of ¹⁴C into glucose by liver, kidney and brain; kidney also formed labelled glucose and serine when incubated with L-[¹⁴C]glutamate. Katz & Chaikoff (1955) have shown that isolated rat-liver slices incubated with labelled acetate incorporate radioactivity into glucose.}

It must be emphasized that the present experiments provide information only about the effects of the uncoupling reagents on the transfer of radiocarbon from the labelled substrates to the soluble intermediates. No attempt has been made to measure the pool size and hence the specific activity of each intermediate and it is therefore not possible to decide if any interference with either the production or utilization of unlabelled material has contributed to the observed effects.

Salicylate produced a number of effects on the incorporation of radioactivity from the labelled pyruvate into the soluble intermediates. Some of these, such as the decreased total ¹⁴C incorporation (Table 1) and the decreased appearance of radiocarbon in glucose (Table 2), were also caused by DNP and were therefore related to an uncoupling action on oxidative-phosphorylation processes. However, in the liver and kidney preparations, salicylate, but not DNP, caused an increased accumulation of radioactivity in glutamic acid which exceeded the values found in the absence of the uncoupling reagents (Table 3). The incorporation of ¹⁴C into glutamic acid in brain in the presence of salicylate was also greater than that which occurred in the presence of DNP. These results suggested that salicylate exerted an effect on glutamic acid metabolism distinct from its uncoupling action.

The principal route for the incorporation of ¹⁴C from the labelled pyruvate into glutamic acid is probably via acetyl-coenzyme A and α -oxoglutarate. The more important pathways involved in the further metabolism of glutamic acid include protein synthesis, decarboxylation to γ -aminobutyric acid and various transamination reactions (Meister, 1957). Both salicylate and DNP inhibit the incorporation of ¹⁴C-labelled amino acids into protein in isolated tissues (Siekevitz, 1952; Manchester, Randle & Smith, 1958) and the present results (Table 2) show that both uncoupling reagents decreased the formation of radioactive γ -aminobutyric acid from the labelled pyruvate. However, 5 mM-salicylate, but not 0.5 mM-DNP, was found to inhibit the activities of the two transaminases involving glutamic acid which are present in rat serum. The effect of salicylate on the serum glutamic-pyruvic-transaminase activity was particularly pronounced and has been confirmed in other work (Steggle, Huggins & Smith, 1961). Manso, Taranta & Nydick (1956) have reported that 0.5–3 mM-salicylate has no effect *in vitro* on either of the two transaminase activities in human serum.

Inhibition of transaminase reactions involving glutamic acid may therefore explain, at least in part, the relative accumulation of radioactivity in the glutamic acid when isolated preparations of rat liver and kidney are incubated with [^{3-¹⁴C]}

pyruvate in the presence of 5 mM-salicylate. An attempt to obtain more direct evidence relating to this hypothesis was made by incubating isolated rat kidney with L-[^{14}C]glutamic acid in the presence or the absence of salicylate or DNP. However, there was little, if any, difference between the effects of the uncoupling reagents. The main changes concerned an inhibition of synthetic reactions, e.g. the incorporation of radioactivity from the labelled glutamate into serine and glucose, and an increased appearance of the isotope into lactic acid, malic acid and fumaric acid. The transfer of ^{14}C from the labelled substrate to γ -aminobutyric acid was decreased in the presence of DNP but salicylate caused a slight increase. No difference between the effects of salicylate and DNP on the amount of residual [^{14}C]glutamic acid was observed. Thus the metabolism of glutamate appeared to have a different sensitivity to salicylate, depending on whether the glutamate was formed from pyruvate or was supplied externally.

SUMMARY

1. The effects of 5 mM-salicylate and 0.5 mM-2,4-dinitrophenol on the incorporation of ^{14}C from [^{3-14}C]pyruvate and L-[^{14}C]glutamate into the soluble intermediates of chopped rat tissues has been studied.

2. In the absence of the uncoupling reagents, the tissue preparations incorporated the isotope into substances known to be involved in the tricarboxylic acid cycle, in transamination and in synthetic reactions.

3. Salicylate and dinitrophenol produced similar effects on the distribution of radioactivity in the

soluble intermediates which appear to be explicable in terms of their uncoupling action on oxidative-phosphorylation reactions.

4. Salicylate, but not dinitrophenol, caused an increased incorporation of ^{14}C from the [^{3-14}C]pyruvate into glutamic acid in liver and kidney and also inhibited the activities of rat-serum glutamic-pyruvic and glutamic-oxaloacetic transaminases.

5. Salicylate and dinitrophenol had similar effects on the distribution of ^{14}C from L-[^{14}C]glutamate in chopped rat kidney.

6. Some implications of these results are discussed.

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Uncoupling Reagents and Metabolism

4. EFFECTS OF SALICYLATE ADMINISTRATION IN THE RAT ON THE INCORPORATION OF ^{14}C FROM LABELLED GLUCOSE AND ACETATE INTO THE SOLUBLE INTERMEDIATES OF ISOLATED LIVER AND BRAIN

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The incubation of salicylate with isolated rat tissues produces effects on the total incorporation and subsequent distribution of ^{14}C from [^{14}C]-

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glucose and [^{2-14}C]acetate in the soluble intermediates, which are explicable in terms of an uncoupling action on oxidative-phosphorylation processes (Smith & Moses, 1960). In the present study, isolated liver and brain preparations were incubated with labelled glucose or acetate after the animals had received either single or repeated