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Uncoupling Reagents and Metabolism

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

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Salicylate uncouples oxidative-phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956; Penniall, Kalnitsky & Routh, 1956; Jeffrey & Smith, 1959; Packer, Austen & Knoblock, 1959). The increased oxygen consumption and diminished amounts of adenosine triphosphate and creatine phosphate observed in the isolated rat diaphragm incubated with salicylate (Smith & Jeffrey, 1956) are directly explicable in terms of this uncoupling action. However, several other reported effects of salicylate on the metabolism of isolated tissues, e.g. the decreased glycogen and protein synthesis produced in rat-liver slices and in diaphragm muscle (Smith, 1955; Manchester, Randle & Smith, 1958), bear a less obvious relationship to uncoupling and could be caused by salicylate acting on enzyme systems other than those involved in oxidative phosphorylation.

The present study is concerned with the effects of salicylate and 2:4-dinitrophenol on the incorporation of ^{14}C from [^{14}C]glucose and [2-^{14}C]acetate into the soluble metabolic intermediates of preparations of isolated rat tissues. The work was undertaken specifically to determine if salicylate produced any effects differing from those of the classical uncoupling agent and more generally to investigate the wider implications of an uncoupling action on tissue metabolism. A preliminary account of this work has been previously published (Smith & Moses, 1960).

EXPERIMENTAL

Preparation of tissues. Male rats (wt. 200–250 g.) of the Wistar strain, maintained on M.R.C. cube diet no. 41, were starved for 24 hr. before being killed by stunning and decapitation. The required tissue (liver, kidney, brain, heart muscle or testis) was removed and placed in ice-cold medium. This medium [based on that used by Randle & Smith (1958) except that the K:Na ratio was altered according to the suggestion of Hastings, Teng, Nesbett & Sinex (1952)] contained (m-moles/l.): K_2HPO_4, 10·1; KCl, 123·0; NaCl, 4·5; Na_2SO_4, 0·3; $CaCl_2$, 1·35; $MgCl_2$, 1·3; glucose 1·0, dissolved in de-ionized water and adjusted to

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pH 7·4 by the addition of HCl. All the tissues, except testis, were then cut into slices nominally 0·728 mm. thick, with a tissue chopper (McIlwaine & Buddle, 1953). The slices were subsequently cut in two directions at intervals of 0·312 mm., to form small blocks. The consistency of testis, after the removal of its capsule, was such that the preliminary slicing procedure was omitted. The approximate wet weights of the tissues initially used for chopping were: liver, 3·5 g.; kidney, 2·0 g.; brain, 1·8 g.; heart muscle, 1·3 g.; testis, 3·9 g. The chopped tissue was suspended in 15 ml. of the medium, centrifuged for 30 sec. at about 90 *g* and the supernatant containing the tissue debris was discarded. This procedure was repeated with the deposit and the tissue was finally resuspended in 10 ml. of medium. All these operations, including the slicing of the tissues, were performed in a room maintained at 0–2°. A sample (2 ml.) of the final tissue suspension was washed several times with water and dried to constant weight at 105° in a tared tube for the determination of the dry weight.

Incubation conditions. Samples of the final tissue suspension (1 ml.) were dispensed from a wide-mouthed pipette into each of six 25 ml. Erlenmeyer flasks. Sodium salicylate or 2:4-dinitrophenol (DNP) solution (0·05 ml.) was added to produce final concentrations of 5 mM-salicylate or 0·5 mM-DNP. In the controls, water was added instead of the uncoupler. A solution of radioactive substrate (0·01 ml.) was then added as either [^{14}C]glucose (10 μc, 0·4 μmole) or [2-^{14}C]acetate (10 μc, 2 μmoles). The ^{14}C-labelled material were obtained from The Radiochemical Centre, Amersham, Bucks. The mixtures were incubated in an atmosphere of air for 3 hr. at 37° in a Dubnoff metabolic shaking machine (H. Mickle, Mill Works, Gomshall, Surrey). Water (5 ml.) was added to each flask 5 sec. before the end of the incubation period, at which time the tissue was separated from the medium by rapid filtration under reduced pressure through a sintered-glass funnel (30 mm. diam. disk; pore size 40–90 μ) containing a layer of Celite filter aid about 1 mm. thick. The reaction flask and tissue were then washed with a further 5 ml. of water and the funnel, which had a rubber stopper attached to its stem, was removed from the filtration flask and inserted into a centrifuge tube so that the rubber stopper formed a seal. Boiling aqueous 80 % (v/v) ethanol (5 ml.) was then poured on to the sintered disk to kill the tissue. About 12 sec. elapsed between filtration of the incubation mixture and the addition of the boiling ethanol. The tissue was allowed to remain in contact with the ethanol for 1 hr. and, after the rubber stopper in the centrifuge tube had been loosened, the ethanolic extract was forced through the filter by compressed air. The funnel was reseated in the centrifuge tube and the tissue further extracted with 2 ml. of boiling 20 % (v/v) ethanol, followed by 2 ml. of boiling water. These extracts were combined and evaporated under reduced pressure at 40° to a final volume of 0·6 ml. The filtrate and washings from the original separation of the tissue, immediately after incubation, were also combined and made up to a volume of 15 ml. with absolute ethanol.

Chromatography. The radioactive substances in the concentrated cell extracts and in the filtered medium were separately analysed by two-dimensional descending paper chromatography, 46 cm. × 57 cm. sheets of Whatman no. 4 filter paper, washed with 1 % (w/v) oxalic acid, being used. Phenol–water (72 %, w/w) was employed as the first solvent and butanol–propionic acid–water (45·9:23·4:30·6, by vol.) as the second (Benson *et al.* 1950). Of the 0·6 ml. of concentrated cell extract obtained in each experiment, 0·2 ml. was chromatographed for 8–10 hr. until the solvents reached the edges of the paper. This chromatogram contained all the substances soluble in aqueous ethanol present in the extract but the various phosphate esters had relatively low R_F values and tended to remain near the origin. The remaining 0·4 ml. of the extract was therefore chromatographed for 24 hr. in each solvent to ensure a more complete separation of the phosphate esters. The high salt content of the filtered medium restricted the quantity which could be analysed by paper chromatography, and 0·5 ml. of the total 15 ml. volume was used, the solvents being allowed to run only to the edges of the paper. The radioactive substances on the chromatogram were located by radioautography with Kodirex X-ray film (Kodak Ltd.). The radioactivity in each spot was counted directly on the chromatogram for 1 min. by means of a Scott type Geiger–Müller tube (Fuller, 1956), flushed continuously with helium which had previously been passed through ice-cold ethanol. Both sides of the chromatogram were counted and the values averaged. The accuracy of each count was approximately proportional to the square root of the number of disintegrations recorded.

The radioactive spots on the chromatogram were identified presumptively by their chromatographic positions and this was confirmed by cochromatography with authentic materials in the same solvent system (Benson *et al.* 1950; Moses, 1960). Phosphate esters were hydrolysed with human prostatic acid phosphatase, prepared by centrifuging the cells from semen and using the supernatant as a source of the enzyme (Schmidt, 1955), and rechromatographed to identify the organic components.

RESULTS

Total incorporation of ^{14}C

The radioactivity measured was that present in the soluble intermediates of the cell extract and medium. The ^{14}C evolved as CO_2 or incorporated into substances, such as glycogen and proteins, which were insoluble in aqueous ethanol, was not estimated. The fate of all the isotope originally present in the labelled glucose or acetate could not therefore be followed. However, an approximate balance of the ^{14}C from the labelled glucose was made by determining the amount of radioactivity remaining as glucose at the end of the experiments. A similar procedure for the [2-^{14}C]acetate was not feasible because of its volatility during chromatography. Brain and testis were found to incorporate 35 % of the total ^{14}C from the labelled glucose into the soluble intermediates, leaving 5 % as unchanged substrate. Thus 60 % of the ^{14}C was liberated as CO_2 or entered ethanol-insoluble substances. Percentages of the ^{14}C incorporated into the soluble intermediates by kidney, heart and liver were 20, 14 and 2 respectively, the remaining isotope being almost completely recovered as unchanged glucose.

The total carbon from the labelled glucose and acetate which was incorporated into the soluble intermediates of the five tissues is given in Table 1.

The least active tissues, liver and kidney, showed proportionally larger incorporations of carbon from acetate than from glucose.

The results in Table 2 show that 5 mM-salicylate and 0·5 mM-DNP inhibited the total incorporation of ^{14}C from the labelled acetate to a much larger extent than that from the labelled glucose. This inhibition was more pronounced for those tissues, liver and kidney, which showed a relatively low glucose:acetate ratio (cf. Table 1). DNP caused some decrease in the incorporation of ^{14}C from [^{14}C]glucose in liver and kidney, whereas both salicylate and DNP decreased the isotope incorporation in testis but increased it in the brain preparation.

Table 1. *Incorporation of carbon from glucose and acetate into the soluble intermediates of rat tissues*

The tissues were cut into blocks and incubated aerobically for 3 hr. at 37° with [^{14}C]glucose or [2-^{14}C]acetate of known specific activities. The unlabelled glucose present in the medium was taken into consideration when calculating the specific activity of the [^{14}C]glucose. The tissues were killed and extracted with ethanol, the extracts were chromatographed and radioactivity was measured in the separated metabolites. From the quantities of ^{14}C incorporated into the metabolites, the total utilizations of glucose and acetate were calculated and are expressed below as μmoles of substrate incorporated into the extract of 25 mg. of dry tissue in 3 hr. The values are calculated from the sum of the ^{14}C present in the metabolites in the cell extract and in the medium.

Tissue	Glucose utilization (μmoles)	Acetate utilization (μmoles)	Glucose utilization / Acetate utilization
Liver	0·07	0·3	0·23
Kidney	0·57	2·0	0·29
Heart	0·50	0·1	5·0
Brain	1·21	0·2	6·1
Testis	2·90	1·1	2·6

Table 2. *Effects of salicylate and 2:4-dinitrophenol on the total incorporation of* ^{14}C *from* [^{14}C]*glucose and* [2-^{14}C]*acetate into the soluble intermediates of rat tissues*

Experimental details were as given in Table 1; salicylate concentration, 5 mM; DNP concentration, 0·5 mM. Results are expressed as percentage inhibitions of ^{14}C incorporations compared with the values observed in the absence of the uncoupling reagents given in Tables 3 and 4. Minus signs indicate stimulation of ^{14}C incorporation.

	[^{14}C]Glucose		[2-^{14}C]Acetate	
Tissue	Salicylate	DNP	Salicylate	DNP
Liver	0	18	92	92
Kidney	1	15	93	91
Heart	0	2	32	32
Brain	−26	−22	70	63
Testis	17	40	60	41

Distribution of ^{14}C *in the soluble intermediates*

The results in Tables 3 and 4 show the amounts of ^{14}C incorporated into the soluble intermediates of the five tissues in the presence and absence of salicylate and DNP.

In the absence of the inhibitors the general pattern of incorporation from both substrates was similar for the five tissues although there was some variation in the percentage distributions of radioactivity in the various fractions. The ^{14}C from the labelled glucose was found in a number of substances known to be involved in established metabolic sequences. The occurrence of radioactivity in the hexose phosphates, phosphoglyceric acid, phosphoenolpyruvic acid and lactic acid is evidence of the glycolytic pathway, in phosphogluconic acid, ribose and sedoheptulose monophosphates is evidence of the hexose monophosphate oxidative sequence and in fumaric acid, malic acid and citric acid is evidence of the tricarboxylic acid cycle. Radioactivity was also detected in various amino acids (glutamic and aspartic) which are derived from α-oxoglutarate and oxaloacetate by transamination reactions. Small amounts of ^{14}C were also detected in adenosine and inosine nucleotides and in uridine diphosphoglucose. Radioactive maltose could not be detected in brain, which incorporated a high proportion of the isotope into the amino and organic acids. Similar results with brain preparations incubated with [^{14}C]glucose have been reported by Chain (1959). The ^{14}C from the labelled acetate appeared mainly in the amino and organic acids but kidney and liver showed radioactivity in the sugar phosphates, phosphoglyceric acid and phosphoenolpyruvic acid and in inosine phosphates. The results were similar to those described by Katz & Chaikoff (1955).

The distribution of ^{14}C from the labelled acetate was much more affected by salicylate and DNP than that from the labelled glucose. The incorporation of the isotope from the acetate was decreased in all the fractions of the soluble intermediates and, when present, was restricted to the amino and organic acids. Relatively little change was observed with the [^{14}C]glucose and there was considerable variation between the tissues. Kidney (Table 3) showed a decreased incorporation of ^{14}C into sugar phosphates, maltose, uridine diphosphoglucose and nucleotides, with no appreciable changes in the amino and organic acids. The increased total incorporation of ^{14}C observed with brain in the presence of the uncoupling reagents

Table 3. *Metabolism of [^{14}C]glucose and [2-^{14}C]acetate by rat liver and kidney in the presence and absence of salicylate and 2:4-dinitrophenol*

Each flask contained 1 ml. of tissue suspension (24·2 mg. dry wt. of liver or 12·3 mg. dry wt. of kidney) in the presence and absence of either 5 mM-salicylate or 0·5 mM-DNP. The tissue was incubated with 10 μC of labelled substrate for 3 hr. at 37°. The table shows the ^{14}C present in each substance in the whole sample, and thus represents the sum of the ^{14}C present in the cell extract and in the medium. Radioactivity is expressed as $10^{-3}\times$ counts/min. per 25 mg. dry wt. of tissue.

	Liver						Kidney					
Labelled substrate ...	[^{14}C]Glucose			[2-^{14}C]Acetate			[^{14}C]Glucose			[2-^{14}C]Acetate		
Uncoupling reagent ...	None	Salicylate	DNP	None	Salicylate	DNP	None	Salicylate	DNP	None	Salicylate	DNP
Sugar diphosphates*	1·4	0·2	0·2	0	0	0	5·5	0·4	0·4	0	0	0
Sugar monophosphates†	6·3	2·2	3·4	0·2	0	0	31	28	16	0·4	0	0
Phosphogluconic acid	0·2	0	0·1	0	0	0	0·2	0	0	0	0	0
Phosphoglyceric acid	1·5	0·3	0·1	2·9	0·2	0·2	3·7	0·6	1·0	0·6	0	0
Phosphoenolpyruvic acid	0·2	0·1	0·05	0·05	0	0·05	1·8	4·9	0·6	0·2	0	0
Uridine diphosphoglucose	1·1	0·1	0·1	0	0	0	3·5	0·6	0·4	0·2	0	0
Adenosine diphosphate	0·5	0·1	0	0	0	0	0·8	0	0	0	0	0
Adenosine triphosphate	0·1	0	0·1	0	0	0	1·6	0	0	0	0	0
Inosine monophosphate	0·2	0	0	0	0	0	0	0	0	0	0	0
Inosine and guanosine diphosphate	0·2	0	0	0·9	0	0	0·2	0	0	0·2	0	0
Inosine triphosphate	0	0	0	0	0	0	0·4	0	0	0	0	0
Maltose	5·1	3·5	6·2	0	0	0	15	2·8	2·8	0	0	0
Fructose‡	4·9	0	5·5	0	0	0	0	42	0	0	0	0
Cysteine	0·3	0	0	0	0	0	0	0	0	0	0	0
Aspartic acid	7·4	8·4	10	12	0·7	0·8	47	35	41	140	11	8·7
Glutamic acid	0·4	0·4	0	18	0·2	0·2	61	28	48	480	33	34
Alanine	13	9·8	15	6·7	0·9	0·8	170	130	100	200	6·7	16
Histidine	0	0	0	1·0	0·2	0	0	0	0	0	0	0
Threonine	0	0	0	1·1	0·7	1·9	0	0	0	44	0	0
Glutamine	0	0	0	6·7	0	0	0	0	0	0	0	0
γ-Aminobutyric acid	0	0	0	1·0	3·1	0	0	0	0	9·4	0·2	0
Glycine + serine§	—	—	—	34	1·2	0	—	—	—	3·9	0	0
Citrulline	0	0	0	0·1	0·3	0·1	0	0	0	0	0	0
Phenylalanine + leucine‖	3·4	7·5	13	1·5	0	3·9	9·6	0	0	0	0	0
Glutathione	0	0	0	0	0	0	0·4	0·4	0	0	0	0
Valine‖	0	1·7	2·3	12	0	0	9·1	49	38	0	0	0
Lactic acid¶	0·6	2·7	0·2	40	0·5	1·8	17	20	28	47	6·9	8·7
Fumaric acid	0	0	0	0·3	0·2	0·2	0·2	0·2	0	0·6	0	0·6
Malic acid	0	0	0	0·7	1·5	0·4	2·8	6·9	9·6	67	4·5	14
Citric acid	0	0	0	0·1	0·1	0·3	0·6	0·6	4·5	0·6	0·4	0·8
Glycollic acid	0	0	0	0	0·9	0·8	0	0	0	0	0	0
Total in:												
Sugar phosphates	9·6	2·8	3·8	3·1	0·2	0·3	42	34	18	1·0	0	0
Uridine diphosphoglucose	1·1	0·1	0·1	0	0	0	3·5	0·6	0·4	0·2	0	0
Nucleotides	1·0	0·1	0·1	1·0	0	0	3·0	0	0	0·2	0	0
Maltose	5·1	3·5	6·2	0	0	0	15	2·8	2·8	0	0	0
Amino acids	24	28	40	95	7·3	7·7	300	250	240	880	52	65
Organic acids	0·7	2·7	0·2	41	3·2	3·4	20	28	42	120	12	24
Unidentified compounds	7·0	2·1	3·5	16	1·0	1·5	23	45	52	25	13	12
Soluble fraction	54	39	59	160	12	13	400	400	350	1000	75	94

* Contains diphosphates of fructose and glucose.
† Contains monophosphates of glucose, fructose, ribose and sedoheptulose.
‡ Possibly a contaminant in the [^{14}C]glucose used as substrate. Its appearance as a separate spot may depend on a variable separation from the residual glucose.
§ Not separated chromatographically. As the two amino acids have chromatographic parameters similar to those of glucose, they are not measured when [^{14}C]glucose is the substrate.
‖ Identity not definitely confirmed by cochromatography with authentic marker.
¶ Identity not definitely confirmed by cochromatography with authentic marker. However, it is chromatographically similar to lactic acid and fairly volatile. The volatility renders the values given above for the amounts of activity in lactic acid unreliable.

Table 4. *Metabolism of [^{14}C]glucose and [2-^{14}C]acetate by rat brain, testis and heart muscle in the presence and absence of salicylate and 2:4-dinitrophenol*

Details were as given for Table 3, except that each flask contained 10·1 mg. dry wt. of brain, 4·4 mg. dry wt. of testis or 9·5 mg. dry wt. of heart muscle.

	Brain						Testis						Heart muscle					
Labelled substrate	[^{14}C]Glucose			[2-^{14}C]Acetate			[^{14}C]Glucose			[2-^{14}C]Acetate			[^{14}C]Glucose			[2-^{14}C]Acetate		
Uncoupling reagent	None	Salicylate	DNP	None	Salicylate	DNP	None	Salicylate	DNP	None	Salicylate	DNP	None	Salicylate	DNP	None	Salicylate	DNP
Sugar diphosphates*	0	0	0	0	0	0	0	0	0	0	0	0	0·8	0·5	2·6	0	0	0
Sugar monophosphates†	24	23	21	0	0	0	130	69	63	0	0	0	26	32	17	0	0	0
Phosphogluconic acid	0·2	0·2	0·5	0	0	0	6·3	11	14	0	0	0	0	0	0	0	0	0
Phosphoglyceric acid	1·0	0·1	0	0	0	0	0	9·7	0	0	0	0	0	0	0	0	0	0
Phosphoenolpyruvic acid	0	0	0	0	0	0	9·7	7·4	9·7	0	0	0	0·5	0·5	0·3	0	0	0
Uridine diphosphoglucose	1·2	0·7	0	0	0	0	17	10	8·5	0	0	0	0	0	0	0	0	0
Inosine triphosphate	0·2	0	0·2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maltose	0	0	0	0	0	0	15	0	34	0	0	0	15	25	13	0	0	0
Fructose‡	31	7·4	36	0	0	0	180	13	100	0	0	0	42	0	0	0	0	0
Aspartic acid	200	220	100	32	6·4	5·0	84	85	76	9·7	4·0	6·3	44	37	38	13	7·4	6·6
Glutamic acid	150	290	210	41	8·7	13	480	370	130	190	46	54	0	0	0	13	6·1	2·1
Alanine	210	190	390	4·2	0	0	470	250	290	0·6	7·4	9·7	26	85	81	0	0	0
γ-Aminobutyric acid	110	83	69	17	5·7	3·7	120	0	0	0	0	0	0	0	0	0	0	0
Glutamine	5·7	0	13	0·2	0	0	0	0	0	16	8·0	0	0	0	0	0	0	0
Cysteine	0·7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glycine + serine§	—	—	—	0	0	0	—	—	—	0	0	0	—	—	—	2·4	0	0·8
Phenylalanine + leucine‖	0	0	0	0	0	0	23	4·0	57	14	0	0·3	24	37	10	19	16	17
Valine‖	6·9	46	50	0	0	0	150	150	110	30	17	27	56	47	45	0	0	0
Lactic acid¶	37	29	58	7·9	2·7	5·9	73	110	61	56	17	48	35	15	40	8·2	11	8·2
Fumaric acid	5·7	6·7	0	0·5	1·0	1·7	0	2·8	0	1·7	0	0	0	0	0	0	0	0
Malic acid	19	28	6·7	6·2	3·5	5·9	13	19	17	17	6·8	22	1·8	8·9	5·3	3·2	0	0
Citric acid	58	150	72	9·7	3·0	3·0	140	110	55	63	41	44	8·9	0	0	0	0	0
Succinic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6·6	7·4	8·9
Total in:																		
Sugar phosphates	25	23	22	0	0	0	150	97	87	0	0	0	27	33	20	0	0	0
Uridine diphosphoglucose	1·2	0·7	0	0	0	0	17	10	8·5	0	0	0	0	0	0	0	0	0
Nucleotides	0·2	0	0·2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Maltose	0	0	0	0	0	0	15	0	34	0	0	0	15	25	13	0	0	0
Amino acids	680	830	830	96	20	22	1300	860	650	270	81	97	150	210	170	48	30	26
Organic acids	120	220	140	24	10	17	220	240	130	140	64	120	45	24	45	18	18	17
Unidentified compounds	6·9	14	13	12	8·4	10	170	300	220	43	32	54	84	130	100	4·4	0	4·6
Soluble fraction	870	1100	1000	130	40	49	2100	1500	1200	450	180	260	360	420	350	70	48	48

* Contains diphosphates of fructose and glucose.
† Contains monophosphates of glucose, fructose, ribose and sedoheptulose.
‡ Possibly a contaminant in the [^{14}C]glucose used as substrate. Its appearance as a separate spot may depend on a variable separation from the residual glucose.
§ Not separated chromatographically. As these two amino acids have chromatographic parameters similar to those of glucose, they are not measured when [^{14}C]glucose is the substrate.
‖ Identity not definitely confirmed by cochromatography with authentic marker.
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(Table 2) was found to involve the amino and organic acid fractions, particularly aspartate, glutamate, alanine and citrate.

DISCUSSION

The results show that chopped preparations of rat tissues incorporate measurable amounts of radioactivity from [^{14}C]glucose and [2-^{14}C]acetate into soluble metabolic intermediates. Thus despite the necessarily large degree of damage undergone by the tissues during their removal from the animal and subsequent preparation, it was possible to show the presence of radioactive substances characteristic of several metabolic pathways. These included glycolysis, the hexose monophosphate oxidative sequence, the tricarboxylic acid cycle and transamination. The majority of radioactive intermediates found were concerned with the catabolism rather than the anabolism of the labelled substrates. This was especially true of the experiments with [^{14}C]glucose. However, ^{14}C from the labelled acetate was found to be incorporated by liver and kidney into phosphoenolyruvate, phosphoglycerate and sugar monophosphates, which indicated the presence of synthetic pathways.

Salicylate (5 mM) and 0·5 mM-DNP had little effect on the total incorporation of ^{14}C from the [^{14}C]glucose into the soluble intermediates of the tissue, but produced a substantial decrease in that from the labelled acetate. This effect was particularly prominent in liver and kidney, which showed the largest incorporation of radioactivity from the acetate compared with that from glucose. The entry of the acetate carbon atoms into the metabolic sequences of the tissues therefore appears to be more dependent on the supply of adenosine triphosphate (ATP) from oxidative-phosphorylation processes than is the entry of the glucose carbon atoms. Unlabelled glucose was present in all the incubation media in addition to the radioactive substrates: the ATP produced during glycolysis, and from other reactions unaffected by salicylate and DNP, appeared to be capable of maintaining the continued entry of glucose but not that of acetate.

The greatly reduced total incorporation of ^{14}C from the [2-^{14}C]acetate in the presence of salicylate and DNP was reflected in the decreased amounts of the isotope found in all fractions of the soluble intermediates. Radioactivity when present was virtually restricted to the amino and organic acids and the participation of the acetate carbon atoms in synthetic reactions in the liver and kidney was negligible. The uncoupling reagents therefore not only decreased the initial uptake of the labelled acetate but also inhibited the conversion of its carbon atoms into substances requiring ATP for their synthesis. These results are compatible with the observation (Smith, 1959) that the incorporation of radioactivity into the liver glycogen of the intact rat given an intraperitoneal injection of [2-^{14}C]acetate is inhibited by the concurrent administration of salicylate.

The distribution of the isotope from the [^{14}C]-glucose among the soluble intermediates indicated that the main catabolic pathways of glycolysis and the tricarboxylic acid cycle were not inhibited by the uncoupling agents. Only very small amounts of isotope were incorporated into phosphogluconic acid and sedoheptulose phosphate in all the experiments, and no conclusions could be drawn about the effects of salicylate and DNP on the hexose monophosphate oxidative pathway. The incorporation of ^{14}C into the amino acids was not affected by the uncoupling reagents, showing that transamination reactions were not inhibited. The only evidence relating to the effects of salicylate and DNP on synthetic pathways was the reduced incorporation of ^{14}C into uridine diphosphoglucose and into the small amounts of nucleotides formed by liver and kidney. The results with the maltose fraction in the five tissues were extremely variable, only the kidney showing a decreased amount of ^{14}C in this fraction in the presence of the uncoupling reagents.

No qualitative differences between the effects of 5 mM-salicylate and 0·5 mM-DNP were detected during the experiments. The general effects of the two substances observed in the present work would appear to be explicable in terms of their uncoupling action on oxidative-phosphorylation reactions in that synthetic reactions involving ATP were inhibited and catabolic reactions not affected.

SUMMARY

1. The effects of 5 mM-salicylate and 0·5 mM-2:4-dinitrophenol on the incorporation of ^{14}C from [^{14}C]glucose and [2-^{14}C]acetate 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 glycolysis, the hexose monophosphate oxidative pathway, the tricarboxylic acid cycle, transamination and synthetic reactions.

3. Salicylate and dinitrophenol considerably reduced the total incorporation of ^{14}C from the labelled acetate, and the subsequent distribution of the isotope was restricted to the amino and organic acid fractions of the soluble intermediates.

4. The total incorporation and distribution of the ^{14}C from the [^{14}C]glucose were not materially altered in the presence of salicylate and dinitrophenol.

5. Some implications of these results are discussed.

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Uncoupling Reagents and Metabolism

2. EFFECTS OF 2:4-DINITROPHENOL AND SALICYLATE ON GLUCOSE METABOLISM IN BAKER'S YEAST

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Studies by Smith & Moses (1960) have shown that most, if not all, of the effects of salicylate and 2:4-dinitrophenol on intermediary metabolism in several animal tissues supplied with labelled glucose or acetate could be explained on the known abilities of these compounds to uncouple oxidative phosphorylation.

The present paper is concerned with similar experiments with baker's yeast. Studies were made of the kinetics of incorporation of the label into different metabolites, samples of the reaction mixtures being taken for analysis at time intervals ranging from a few seconds to several hours after the addition of the labelled substrate. A preliminary report of this work has already been published (Moses & Smith, 1960).

EXPERIMENTAL

Block yeast (3 g.) (The Distillers Co. Ltd.) was suspended in water and centrifuged at 1600 *g* for 5 min. The volume of the wet-packed cells was 3·8 ml. Half the cells (starved cells) were suspended in 25 ml. of tap water and the other half ('growing' cells) in 25 ml. of the following medium (g./l.); glucose, 10; NH_4Cl, 2; KH_2PO_4, 5·44; $MgSO_4,7H_2O$, 0·1; NaCl, 0·1, dissolved in London tap water [containing approximately (p.p.m.): Ca, 100; Na, 20–25; K, 5; Mg, 5 (all as bicarbonates); free Cl_2, less than 0·05] and adjusted to pH 5 with KOH. Each suspension was shaken at 25° for 3 hr., the cells were centrifuged, washed twice with 2 mM-KH_2PO_4, adjusted to pH 5 with KOH and resuspended in 19 ml. of this solution, which resulted in a cell concentration of 100 μl. of wet-packed cells/ml. When required, the final suspension also contained 2:4-dinitrophenol (DNP) or sodium salicylate at the appropriate concentration.

For the study of kinetics, 15 ml. of cell suspension was placed in a 50 ml. round-bottom flask equipped with a standard joint into which was fitted a Kipps automatic tilt pipette [H. J. Elliott Ltd. (E-Mil), Pontypridd, Glam.] calibrated to deliver 1 ml. The flask was shaken in air at 25° and the reaction was started, after removal of the tilt pipette, by the addition of 150 μl. of a solution of [^{14}C]-glucose (37·5 μc; 1·5 μmole; final glucose concn. 0·1 mM). [^{14}C]Glucose was obtained from The Radiochemical Centre, Amersham, Bucks.

The tilt pipette was replaced immediately and samples (1 ml.) of the suspension were run directly into 4 ml. of ethanol at room temperature at the following times after the addition of the labelled substrate: 5, 10, 15, 30, 45, 60,

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