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SHORT COMMUNICATIONS

The effects of salicylate on guinea-pig testis mitochondria compared with the effects of aging and repeated washing

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SALICYLATE, γ-resorcylate and gentisate have been reported to inhibit many dehydrogenase and transaminase enzymes.[1, 2] The mechanism of inhibition for malate and isocitrate dehydrogenases involves competition with the relevant coenzyme.[3, 4] In addition gentisate has been shown to be a potent inhibitor of rat liver mitochondrial succinoxidase *in vitro*.[5] These inhibitory effects were first observed as modifications of two dimensional chromatograms obtained after incubating various sub-cellular preparations with radioactive substrates, and were subsequently confirmed in the specific isolated enzyme system. The use of the chromatographic technique as a drug-screening tool was thus readily established. The present communication seeks to point out a possible pitfall in evaluating drug effects by this technique.

Mitochondria obtained from 10 g wet wt. of decapsulated guinea-pig testes were suspended in 2·5 ml, 0·25M sucrose at 0–4°, and aliquots (50 μl) taken for experimental purposes. Sedimentation by centrifugation, and resuspension was carried out a further five times, with the sucrose volume for each resuspension being reduced by the total volumes used in the previous assays. Each aliquot was incubated aerobically, with shaking, for 36 min at 37° with 50 μl of a solution containing cytochrome *c* (0·03 mM), ADP (4·0 mM), ATP (1·0 mM), KCl (5 mM) and [1:4-$^{14}C_2$] succinate (0·75 μc; 0·03 μmole), dissolved in 0·1 M potassium phosphate buffer, pH 7·4. The reactions were stopped by addition of 400 μl of boiling absolute ethanol and the mixtures centrifuged at 500 *g*. The radioactive substances present in the supernatants were separated by two dimensional paper chromatography, visualised by auto-radiography and their radioactivity measured by the techniques described by Smith and Moses.[6]

RESULTS AND DISCUSSION

Table 1 shows the effect of repeated sedimentation and resuspension on mitochondrial metabolism. The total incorporation of radioactivity into organic acids progressively increased while that in the amino acids decreased, these two observations seem to be complementary. The distribution of radioactivity between the soluble intermediates shows that the main effect of repeated washing is to interfere with the further metabolism of malate and the synthesis of aspartate. The total nitrogen content of these mitochondrial suspensions decrease after each wash. The three hydroxy benzoates produced a marked alteration in the control pattern (Table 3), but caused no observable inhibition in mitochondria which had been washed more than three times. Table 2 shows that there was little change in the distribution of radioactivity in mitochondrial suspensions which had been stored for more than two hours in the cold room (0–4°).

The results show that testis mitochondrial preparations incorporate radiocarbon from [1:4-$^{14}C_2$] succinate into intermediates of the tricarboxylic acid cycle (fumarate, malate, citrate, α-ketoglutarate), substances associated with the cycle (aspartate, asparagine and glutamate), and into alanine and lactate. This qualitative pattern was not altered by any of the experimental procedures nor by the action of inhibitors. Quantitative relationships were altered, however. Repeated washing, and exposure to salicylate, γ-resorcylate and gentisate had similar effects on the incorporation of radioactivity into organic and amino acids. The ultilization of the labelled succinate remained approximately the same throughout the aging and washing experiments, which agrees with the findings of Hogeboom *et al.*[7] The build up of fumarate, malate and citrate with repeated washing was presumably due to the loss of intra-mitochondrial NAD and NADP. Lester, *et al.*,[8] and Hunter and Schultz[9] have demonstrated that NAD is lost from ageing mitochondria, the present results suggest that this process does not cause significant changes in mitochondrial metabolism over a period of about 2 hr.

TABLE 1. THE DISTRIBUTION OF RADIOACTIVITY FROM [1:4-$^{14}C_2$] SUCCINATE IN THE SOLUBLE METABOLIC INTERMEDIATES OF GUINEA-PIG TESTIS MITOCHONDRIA SUBJECTED TO REPEATED SUSPENSION AND SEDIMENTATION: WITH NITROGEN ESTIMATIONS

Soluble intermediates	Number of suspensions and sedimentations					
	1	2	3	4	5	6
Fumarate	3·0	6·7	8·1	9·8	11·6	12·4
Malate	9·8	15·4	24·8	33·1	40·3	45·4
Citrate	4·7	5·4	5·5	4·0	4·1	3·1
α-ketoglutarate	0·2	0·2	0·2	0·2	0·1	0·3
Aspartate	24·5	13·1	6·5	5·2	5·1	4·4
Asparagine	0·2	0·8	1·1	1·0	0·8	0·6
Glutamate	3·3	1·7	1·0	0·8	0·8	0·6
Alanine	0·2	0·3	0·2	0·2	0·1	0·1
Lactate	0·5	0·4	0·3	0·5	0·7	0·6
Residual Succinate	6·0	2·7	3·5	4·4	4·1	3·9
Total in:						
Amino acids	28·2	15·9	8·8	7·2	6·8	5·7
Organic acids (excluding residual succinate)	18·2	28·1	38·9	47·6	56·8	61·8
*Nitrogen (μg/50 μl sample)	239	143	103	95	80	70

Results expressed as counts/min × 10^{-3}. All observations reported here are the mean of at least four separate estimations.

* Nitrogen assays by the method of Kabat and Mayer.[11]

TABLE 2. THE DISTRIBUTION OF RADIOACTIVITY FROM [1:4-$^{14}C_2$] SUCCINATE IN THE SOLUBLE METABOLIC INTERMEDIATES OF AGED MITOCHONDRIA FROM GUINEA-PIG TESTIS: WITH NITROGEN ESTIMATIONS (In these experiments mitochondria from the second suspension were stored at 0–4° in 0·25 M sucrose without further washing)

Soluble intermediates	Control	Mitochondrial suspension allowed stand for 140 min at 4°	
		A	B
Fumarate	6·7	6·4	6·7
Malate	15·4	19·8	18·5
Citrate	5·4	4·9	4·4
α-keto-glutarate	0·2	0·3	0·2
Aspartate	13·1	11·9	12·8
Asparagine	0·8	1·2	1·1
Glutamate	1·7	1·4	1·2
Alanine	0·3	0·1	0·2
Lactate	0·4	0·2	0·7
Residual succinate	2·7	2·9	1·8
Total in:			
Amino acids	15·9	14·6	15·3
Organic acids (excluding residual succinate)	28·1	31·6	30·5
Nitrogen (μg/50 μl sample)	143	135	138

Results expressed as counts/min × 10^{-3}.

The rapid decline of the incorporation of radioactivity into aspartate is presumably due to the reduced availability of oxalo-acetate derived from malate.

An alternative suggestion is that other amino acids which could serve as substrates for transamination leak out of the mitochondria during the washing process. Evidence for this is to be found in the drop in total nitrogen in the preparations during the experiment, so that it seems likely that both processes were operating. In the aging experiment no such drop in nitrogen content was observed. Similar results were obtained in the presence of the drugs. All three hydroxy benzoates caused in-

TABLE 3. THE EFFECTS OF SALICYLATE AND SOME SALICYLATE CONGENERS ON THE INCORPORATION OF RADIOCARBON FROM [1:4-$^{14}C_2$] SUCCINATE IN THE SOLUBLE INTERMEDIATES OF GUINEA PIG TESTIS MITOCHONDRIA

(The salicylate, γ-resorcylate and gentisate were added to the solution of cofactors to give a final concentration of 5 mM)

Soluble intermediate	Control	Salicylate (5 mM)	γ-resorcylate (5 mM)	Gentisate (5 mM)
Fumarate	6·7	9·8	9·1	8·3
Malate	15·4	22·0	19·6	17·2
Citrate	5·4	7·4	8·1	5·8
α-ketoglutarate	0·2	0·2	0·2	0·1
Aspartate	13·1	8·9	5·3	5·6
Asparagine	0·8	0·6	0·1	0·3
Glutamate	1·7	1·0	0·7	0·9
Alanine	0·3	0·2	0·2	0·2
Lactate	0·4	0·2	0·3	0·4
Residual succinate	2·7	5·6	9·5	8·2
Total in:				
Amino acids	15·9	11·7	6·3	7·0
Organic acids (excluding residual succinate)	28·1	39·4	37·0	31·4

Results expressed as counts/min × 10^{-3}.

creases of the radioactivity in fumarate, malate and citrate and a decrease of radioactivity in aspartate. In this case, however, the changes can be ascribed to competition with NAD and NADP and inhibition of transaminase activity.[3, 10] Thus, entirely different processes bring about a similar end result. In the present instance the results obtained from repeated washing reproduce closely the effects of salicylate and its congeners. Although, mitochondria which are washed several times during isolation are not effected by any of the drugs. It is of considerable importance in the assaying of chemicals for drug action in sub-cellular fractions that changes which may be due to slight differences in handling should not be ascribed to an action of the drug itself.

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