STUDIES ON ALPHA OXIDATION OF STEARIC ACID BY TETRAHYMENA PYRIFORMIS AND CRITHIDIA FASICULATA

L. R. Avins*

Biological Laboratory, Amherst College

Amherst, Massachusetts 01002

Received May 22, 1968

Metabolism of fatty acids by alpha oxidation occurs in mammals (Fulco and Mead, 1961), and failure of normal alpha oxidative mechanisms is associated with Refsum's syndrome, a neurological disease of man (Steinberg, 1967). Whether alpha oxidation is important or even exists in lower animals is unknown. The present study was undertaken to seek the presence of an alpha oxidative pathway in the protozoa Tetrahymena pyriformis and Crithidia fasiculata, employing the differential inhibitory effects of malonate upon the beta oxidative pathway (Quastel and Woolridge, 1928), and of imidazole upon the alpha oxidation (Hitchcock and James, 1966). The results suggest the presence of both alpha and beta oxidative pathways in these organisms.

Materials and Methods

Pure 400 ml cultures of each organism were raised for 36 hours at 22° C and then harvested by centrifugation at 3000 rpm for 5 minutes. Cells were resuspended in 20 ml incubation medium containing 0.06 ml 1-^{14}C-stearic acid (37.1 mC/mM, New England Nuclear Corp.) diluted 1:18

* Fellow on USPHS Training Grant (5 TI GM 596) in Microbiology, G. W. Kidder, Program Director.

in benzene. The final concentration of stearic acid in all incubation media was 3.39×10^{-6} M, corresponding to an addition of 0.042 uC/3 ml suspension. All media were autoclaved prior to use. Incubations of 3 ml of the resuspended cells were carried out with shaking at 22° C in Warburg flasks containing 0.3 ml Hyamine hydroxide (Rohm and Haas Chemical Corp.) in the center well and 0.06 ml 5 N H_2SO_4 in one sidearm. Acid was tipped in at the conclusion of a run to liberate dissolved CO_2, and shaking continued for 2 hours to ensure optimal recovery of label. Hyamine containing the labeled carbon was removed from the center well of the Warburg flask to a glass counting vial containing 0.63 ml "Liquifluor" scintillator fluid (New England Nuclear Corp.) in 14.4 ml toluene. The efficiency of this method of $^{14}CO_2$ trapping exceeds 85% (Cuppy and Crevasse, 1963). Using a Packard 3003 liquid scintillation counter, a sufficient number of counts above background were obtained to ensure significance $\pm$5% at the 95% confidence level. Net counts were divided by the counting period to obtain counts per minute as presented in Tables I and II.

In determining labeled acetate, volatile acids were trapped by addition of NaOH from the sidearm of the Warburg flask at the conclusion of an experiment. Dissolved CO_2 was precipitated by addition of $BaCl_2$. Incubation mixtures were subjected to ultrasonic treatment (Measuring and Scientific Equipment, Ltd.) with microglass beads added to facilitate cellular disruption. The absence of whole cells in the sonicated mixture was verified microscopically, and the cell-free suspension acidified prior to distillation to a cooled receiver containing 0.5 ml 5 N NaOH. An equal volume of water was added to the distilling flask and the distillation repeated. Distillates were filtered through acid washed "Celite" (Johns Manville Corp.) and concentrated *in vacuo* to a final known volume of 5-10 ml. For liquid scintillation counting, 0.5 ml of the concentrated distillate was added to 14.5 ml scintillator fluid.

Radioactive material in the distillates was identified as acetate and stearate by paper chromatography. Chromatograms were developed descendingly according to the method of Brown (1950) in ethanol:butanol: ammonia (5:4:1::v:v:v). Two radioactive peaks were identified as acetate and stearate using a strip scanner and labeled standards. Four such distillations each with paired determinations of $^{14}CO_2$ production were performed for Tetrahymena and Crithidia.

Results

The results of incubation with 10 mM malonate are summarized in Table I. Malonate depressed $^{14}CO_2$ production by 29% in Tetrahymena and by 57% in Crithidia. In Tetrahymena an increase of 161% in the pool of recoverable labeled acetate was observed. In Crithidia an increase of 94% was found.

The results of incubation with 10 mM imidazole are summarized in Table II. Imidazole inhibited $^{14}CO_2$ production by 39% in Tetrahymena and by 50% in Crithidia. Unlike malonate, however, this inhibitor had no significant effect upon the pool of labeled acetate in these organisms (see Discussion).

Table I

Effect of 10 mM malonate upon 2 hour $^{14}CO_2$ and $CH_3{}^{14}COOH$ production by Tetrahymena and Crithidia incubated with 1-^{14}C-stearate

CPM/flask

	$^{14}CO_2$			$CH_3{}^{14}COOH$		
	no add'n	malonate	change	no add'n	malonate	change
Tetrahymena	38	27	-29%	97	242	+161%
Crithidia	69	30	-57%	102	198	+94%

Substrate concentration = 3.39×10^{-6} M; 0.042 uC/3 ml incubation medium.

Table II

Effect of 10 mM imidazole upon 2 hour $^{14}CO_2$ and $CH_3{}^{14}COOH$ production by Tetrahymena and Crithidia incubated with 1-^{14}C-stearate

CPM/flask

	$^{14}CO_2$			$CH_3{}^{14}COOH$		
	no add'n	imidazole	change	no add'n	imidazole	change
Tetrahymena	135	83	-39%	242	284	+18%
Crithidia	169	85	-50%	147	135	-8%

Substrate concentration = 3.39×10^{-6} M; 0.042 uC/3 ml incubation medium.

Discussion

The inhibitory effect of malonate is based upon its irreversible combination with succinic dehydrogenase (Quastel and Woolridge, 1928). In so blocking the tricarboxylic acid cycle, malonate prevents the oxidation of acetate, the principal product of the beta oxidation, to CO_2. The finding in both Tetrahymena and Crithidia of depressed $^{14}CO_2$ production with sharply increased levels of 1-^{14}C-acetate therefore suggests a beta oxidation of the substrate by these organisms.

Imidazole is a specific inhibitor of the alpha oxidation in a variety of plant systems and the evidence suggests that the plant and animal systems investigated to date are similar if not identical (Hitchcock and James, 1966). The inhibitory effect of imidazole upon $^{14}CO_2$ production is essentially unaccompanied by changes in 1-^{14}C-acetate. The changes in recoverable 1-^{14}C-acetate are due to a small variation in the efficiency of the distillation procedure and are not significant.

These findings with imidazole are consistent with two hypotheses: (1) inhibition by imidazole of the beta oxidation prior to the cleavage of acetate, and (2) inhibition by imidazole of the alpha oxidation as

previously reported (Hitchcock and James, 1966). There is no evidence that imidazole affects reactions other than the alpha oxidation, and it is concluded that Tetrahymena and Crithidia probably contain the machinery for the alpha oxidation of fatty acids.

References

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Acknowledgment

The author wishes to thank Professor G. W. Kidder for his invaluable advice and encouragement.