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Metabolism of Propionate by Sheep Liver

PATHWAY OF PROPIONATE METABOLISM IN AGED HOMOGENATE AND MITOCHONDRIA

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Experiments were conducted with aged nuclear-free homogenate of sheep liver and aged mitochondria in an attempt to measure both the extent of oxidation of propionate and the distribution of label from [2-^{14}C]propionate in the products. With nuclear-free homogenate, propionate was 44% oxidized with the accumulation of succinate, fumarate, malate and some citrate. Recovery of ^{14}C in these intermediates and respiratory carbon dioxide was only 33%, but additional label was detected in endogenous glutamate and aspartate. With washed mitochondria 30% oxidation of metabolized propionate occurred, and proportionately more citrate and malate accumulated. Recovery of ^{14}C in dicarboxylic acids, citrate, α-oxoglutarate, glutamate, aspartate and respiratory carbon dioxide was 91%. The specific activities of the products and the distribution of label in the carbon atoms of the dicarboxylic acids were consistent with the operation solely of the methylmalonate pathway together with limited oxidation of the succinate formed by the tricarboxylic acid cycle via pyruvate. In a final experiment with mitochondria the label consumed from [2-^{14}C]propionate was entirely recovered in the intermediates of the tricarboxylic acid cycle, glutamate, aspartate, methylmalonate and respiratory carbon dioxide.

In a previous study of propionate metabolism in sheep-liver homogenates (Smith & Osborne-White, 1965) it was found that, though nuclear-free homogenates lost little (5%) of their capacity to metabolize propionate on aging for 19min. without added substrate, washed mitochondria on similar treatment lost about 60% of their activity. Even without aging, mitochondria metabolized propionate at only about 40% of the rate of the nuclear-free homogenates from which they were derived. It was subsequently shown (Smith, Osborne-White & Russell, 1965*a*,*b*) that the protective and stimulatory effects of the soluble fraction of the homogenate on mitochondrial propionate metabolism lay chiefly in the presence of L-glutamate, but partly in the presence of a protein fraction. The soluble fraction of the homogenate alone did not metabolize propionate. Glutamate could be replaced by α-oxoglutarate, and further work has shown that the activity of aged mitochondria may be largely restored to the initial level by the addition, after aging, of low concentrations (1·67mM) of α-oxoglutarate (or glutamate) (Smith & Russell, 1967).

The present work allows a comparison of the products of propionate metabolism by aged nuclear-free homogenate and aged mitochondria. It was undertaken for two reasons: first, to detect any changes in the nature of propionate metabolism that arose as a result of aging the mitochondria without the soluble fraction; secondly, to examine, from the accumulation of products, the extent to which propionate was oxidized by aged mitochondria and nuclear-free homogenate.

The latter question arose from the observation (Smith & Osborne-White, 1965) that a close correlation existed between the rates of consumption of propionate and oxygen by aged nuclear-free homogenates, the oxygen consumed per unit of time being equivalent to the complete oxidation of half the propionate metabolized. This was consistent with the quantities of dicarboxylic acids that accumulated (57% of the propionate consumed on a molar basis), but no explanation of the correlation could be found. Pennington & Sutherland (1956) had previously shown that up to 50% of the propionate consumed by slices of sheep rumen epithelium accumulated as lactate.

Evidence was presented (Smith & Osborne-White, 1965) that propionate was metabolized by the methylmalonate pathway (Flavin & Ochoa, 1957;

Cannata, Focesi, Mazumder, Warner & Ochoa, 1965), but the possibility remained that a second pathway of propionate metabolism may have accounted for the oxidized fraction of the propionate metabolized. Willmer & Gutfreund (1963), on the basis of the insensitivity to malonate of the conversion of propionate into malate, had postulated a second major pathway in guinea-pig mammary-gland slices. Pennington (1954) and Pennington & Sutherland (1956) had previously noted that propionate metabolism by slices of sheep rumen epithelium was relatively insensitive to inhibition by malonate.

The present paper describes experiments in which attempts were made to measure quantitatively the accumulation of the products of [2-^{14}C]propionate metabolism by aged nuclear-free homogenate and aged mitochondria, and at the same time to account quantitatively for the ^{14}C. Additional evidence of the pathway of propionate metabolism was sought by degradation of the major products. Although the attempt to measure both the extent of oxidation and the complete distribution of ^{14}C among products was not entirely successful in any single experiment, the results obtained leave little doubt that the metabolism of propionate by sheep-liver mitochondria, under the conditions used, proceeded virtually exclusively by the methylmalonate pathway and the tricarboxylic acid cycle. The results also show that the degree of oxidation of propionate observed in nuclear-free homogenates was not essential for propionate metabolism to occur. Mitochondria were capable of sustaining propionate consumption without such extensive oxidation and so there was no simple connexion between oxygen consumed and propionate consumed. However, a connexion may have existed between the greater extent of oxidation by nuclear-free homogenate and the higher rate of propionate consumption due to the capacity of the more highly oxidized intermediates to stimulate the rate catalytically.

MATERIALS AND METHODS

Materials. Sodium [2-^{14}C]propionate was obtained from The Radiochemical Centre, Amersham, Bucks. After dilution to 100 μmoles with unlabelled propionic acid, it was chromatographed on a Celite column as described under 'Column chromatography', and the appropriate fractions were steam-distilled before further dilution with unlabelled fractionally distilled propionic acid to the required specific activity. L-Malic acid obtained from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A., was employed without further purification. It was 99% pure by titration and 96% pure by estimation with malate dehydrogenase by the method of Hohorst (1963), and the optical rotation in the presence of uranyl acetate indicated the absence of D-malic acid (Dakin, 1924). Cytochrome *c* (from horse heart, type III), ATP and DL-isocitric acid lactone were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. The lactone was hydrolysed for 20 min. at 60° in excess of KOH and neutralized before use. Celite (Hyflo Super-Cel from Johns–Manville Co., New York, N.Y., U.S.A.) was soaked overnight in 3N-HCl, packed in a column and washed with water until free of acid. While still wet it was washed on the column with 2 bed vol. of redistilled diethyl ether, then spread on a tray and dried in an oven at 110°. Commercial chloroform (B.P., 2l.) was mechanically emulsified with 500 ml. of 1% (w/v) NaOH for 5 min., separated and distilled. Butan-1-ol (2·5l. of laboratory-reagent grade from British Drug Houses Ltd., Poole, Dorset) was refluxed for 1 hr. with 5g. of NaOH and 10 ml. of water; 50g. of anhydrous K_2CO_3 was added and the mixture shaken. After standing overnight the solvent was decanted and distilled. L-Aspartic acid and L-glutamic acid (British Drug Houses Ltd.) were recrystallized from water. Oxaloacetic acid and α-oxoglutaric acid were obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Sodium pyruvate was obtained from E. Merck A.-G., Darmstadt, Germany. Other materials were as described by Smith & Osborne-White (1965) or were analytical-grade reagents. Glass-distilled water was used throughout.

Animals used were Australian Merino ewes, 4 years of age, which were removed from pasture 14 days or more before slaughter and fed *ad libitum* on a diet of 50% of wheaten hay chaff and 50% of lucerne hay chaff (by weight). Nuclear-free homogenates and twice-washed mitochondria were prepared in 0·25M-sucrose from the livers of freshly killed animals as described by Smith *et al.* (1965*a*).

Incubations and preparation of samples. The medium in which homogenates were incubated had the following final composition: K_2HPO_4 (16·4mM), KH_2PO_4 (3·6mM), $NaHCO_3$ (25mM), KCl (85mM), $MgCl_2$ (5mM), sucrose (42mM), ATP (1·3mM), cytochrome *c* (11 μM), potassium [2-^{14}C]propionate (5mM, added after 19 min. in the bath at 37°) and homogenate or mitochondria as described in the text. The medium was in equilibrium at 37° with O_2+CO_2 (95:5) at pH 7·4.

Incubations in Expts. 1 and 2, with nuclear-free homogenate and washed mitochondria respectively, were carried out in 1l. conical flasks connected via taps to 200 ml.-capacity CO_2 traps; the apparatus was similar in design to that described by Pennington & Sutherland (1956). Flasks were flushed with gas mixture (O_2+CO_2, 95:5), pregassed medium (154 ml.) was added to each, and the flasks were shaken and flushed for a further 30 min. (5l. of gas mixture/min. through each flask). Ice-cold nuclear-free homogenate or mitochondria (35 ml.) was then added from a syringe via a polyethylene tube (internal diam. 1 mm.) fitted to a stainless steel tube of similar diameter projecting into the flask through a rubber stopper fitted to a side arm. The flask was then incubated with shaking (80 cyc./min.) and gassing (5l./min.) for 19 min. before adding substrate. Substrate solution (21 ml., with 1050 μmoles of [2-^{14}C]propionate, 19·1 and 17·5 mμc/μmole in Expts. 1 and 2 respectively) containing buffer, KCl, sucrose and $MgCl_2$ at the final concentrations, and already saturated with gas mixture at 37°, was added by syringe and the flask sealed. Incubation with shaking at the same rate was continued for 36 min., and the reaction was stopped by addition by syringe of 43 ml. of 0·66N-H_2SO_4. At the same time the taps to the CO_2 trap were opened and the flask was flushed with N_2 gas (3l./min.)

for 35min. at 37° with shaking. The CO_2 trap contained 200ml. of 2N-NaOH and between the flask and the trap was fitted a short (6in.) Allihn condenser through the jacket of which ice-cold water was circulated throughout the experiment. During the initial gassing (which was diverted from the CO_2 trap) this condenser accumulated a few millilitres of water, which subsequently acted as a trap for any propionic acid volatilized during recovery of $^{14}CO_2$. The water was later rinsed back into the incubation flask. Precipitation of protein was completed by addition of 35ml. of 10% (w/v) $Na_2WO_4,2H_2O$ through the polyethylene tube followed by brief shaking. The contents of the flasks were centrifuged, and the sedimented protein was washed twice by resuspending in 50ml. of water and recentrifuging. After the volume had been measured the combined supernatants were stored at −20°. In Expts. 1 and 2, duplicate flasks were incubated, and initial values for propionate and the products of its metabolism determined from similar duplicate flasks in which protein was precipitated at the time of addition of substrate.

Expt. 3 was performed in 22 replicate tubes, each containing a final volume of 6ml. including 1ml. of washed mitochondria (1·93mg. of N) derived from 0·33g. of liver. The experiment was aimed at the complete recovery in labelled intermediates and respiratory CO_2 of the ^{14}C consumed as [2-^{14}C]propionate. The conditions of incubation were similar to those described by Smith *et al.* (1965*a*), except that, after the preliminary 19min. incubation at 37° with gassing, gas flow was stopped by sealing the exit vent with a rubber stopper or serum cap and the inlet tube with a spring clip. Labelled substrate was then added from the side arms of all flasks simultaneously and the incubation continued with rapid shaking (120cyc./min.) for 36min. Reactions were stopped and protein was precipitated with $Zn(OH)_2$ after the preliminary treatments outlined below.

Six of the tubes contained glass cups (about 1·5ml. capacity) supported on 4cm. glass rods. These were employed to collect respiratory $^{14}CO_2$. At the conclusion of incubation these tubes were removed to a second rack, 0·5ml. of 30% (w/v) NaOH was added to the cup by syringe through the serum cap and 0·5ml. of 5N-H_2SO_4 was added in the same way to the flask contents. Incubation of these tubes was continued at 37° with shaking for 30, 60 or 90 min., when the tubes were opened and the contents of the cups recovered for estimation of $^{14}CO_2$. Maximum recovery of $^{14}CO_2$ was achieved in 60min. and the values quoted are the means of the duplicate 60min. and 90min. recoveries.

The rack with all the remaining tubes was transferred immediately on conclusion of the incubation to an ice–water bath, the tubes were chilled rapidly and opened, and 500mg. of the appropriate carrier (as neutral sodium salt in 6ml. of solution) was added before the addition of protein precipitant. Where only residual volatile acid was determined, protein precipitant was added at once. The tubes to which methylmalonic acid carrier was added were made alkaline with 1ml. of 5N-NaOH and incubated at 50° for 1hr. to hydrolyse methylmalonyl-CoA, and 1ml. of 5N-H_2SO_4 was added followed by the protein precipitant. After addition of $Zn(OH)_2$, water was added to a final volume of 20ml. (except to tubes containing keto acid carriers). The suspensions were centrifuged to remove protein, and the clear supernatant was stored at −20°. Tubes containing carrier α-oxoglutaric acid, pyruvic acid or oxaloacetic acid, after precipitation and removal of protein, were acidified with 5ml. of 5N-HCl, and 40ml. of 0·25M-phenylhydrazine hydrochloride in N-HCl was added to precipitate phenylhydrazones. After refrigeration overnight these were recovered by centrifugation and washed with 10ml. of water.

After extraction of carrier succinic acid from the acidified solution with diethyl ether, carrier glutamic acid was precipitated as zinc glutamate by the addition of excess of zinc acetate to the solution, after neutralization with ZnO and NaOH. Zinc glutamate crystallized after boiling for 30min. and chilling for 2hr. The crystals were washed with water and dissolved in hot 10% (v/v) acetic acid, and Zn^{2+} was removed with H_2S. The clear supernatant was evaporated to dryness *in vacuo*.

Carrier aspartic acid, which was recovered after extraction of carrier fumaric acid from the acidified solution with diethyl ether, was precipitated as copper aspartate by the addition of excess of copper acetate to the neutralized solution and boiling. Copper aspartate was centrifuged down and washed repeatedly with water. After dissolving in hot 20% (v/v) acetic acid, Cu^{2+} was removed with H_2S and the colourless solution evaporated to dryness *in vacuo*.

All other carriers were recovered by extraction overnight of the acidified (pH below 2, with H_2SO_4) solutions with freshly distilled peroxide-free diethyl ether in liquid/liquid extractors. After evaporation of the ether, the acids were recrystallized.

All carriers were recrystallized at least four times, samples at each stage desiccated and the specific activities determined by scintillation counting. Weighed samples (about 5mg.) were dissolved in 5ml. of Bray's (1960) liquid-scintillator solution and counted as described below. Malic acid and methylmalonic acid were recrystallized five times and citric acid was recrystallized six times. All substances except L-malic acid reached constant specific activity, generally in three crystallizations. Malic acid continued to lose activity up to the fifth crystallization, when insufficient sample remained for further work. The loss in specific activity of malic acid between the third and fourth crystallizations was about 19%, that between the fourth and fifth about 4%. The value for ^{14}C in L-malic acid may therefore be overestimated. Duplicate samples of the final crystals from each carrier were burned and the specific activities determined on the $^{14}CO_2$ as described under 'Estimation of ^{14}C'.

The solvents from which the carriers were crystallized were as follows: succinic acid, fumaric acid, L-glutamic acid and L-aspartic acid from water; methylmalonic acid and L-malic acid from acetone–benzene; citric acid twice from diethyl ether–chloroform, twice from ethanol–chloroform and twice from acetone–benzene; α-oxoglutaric acid phenylhydrazone from ethanol–water (1:4, v/v); pyruvic acid phenylhydrazone from ethanol; oxaloacetic acid phenylhydrazone from methanol–diethyl ether (1:1, v/v) plus *n*-hexane to cloudiness. The phenylhydrazones of both oxaloacetic acid and α-oxoglutaric acid formed anhydrides on desiccation, as indicated both by their melting points and by carbon recovery on combustion. The results have been treated accordingly.

Column chromatography. The labelled products in Expts. 1 and 2 were recovered by column chromatography of the major part of the protein-free supernatant. The method used was a modification of that of Swim & Krampitz (1954) with a Celite column (40cm. × 1·2cm. diam.) containing

20g. of washed Celite and 8ml. of 0·2N-H_2SO_4, prepared with a mobile phase of chloroform. The sample was acidified to pH below 2 by dissolving in 1ml. of 3N-H_2SO_4, mixed in a flask with 2·5g. of dry Celite and a little chloroform, and applied to the column as a cap. The column was eluted first with 400ml. of chloroform, then successively with 200ml. each of chloroform containing 10%, 20%, 30% and 40% (v/v) of butan-1-ol. All solvents were previously equilibrated with 0·2N-H_2SO_4. Fractions (212 of 10ml.) were collected and, after removal of 0·1ml. for preliminary estimation of ^{14}C by scintillation counting, were titrated under N_2 with 0·1N-NaOH (carbonate-low).

In preliminary tests of recoveries of added acids from a simulated incubation medium on the same scale it was found that losses of citric acid and isocitric acid, and to a smaller extent of malic acid, occurred if precipitation of salts (particularly magnesium phosphate) was allowed to occur on preliminary concentration of the protein-free supernatant. The solutions were therefore first washed through a column containing 50ml. of Dowex 50 (H^+ form) to remove cations, neutralized with NaOH and concentrated *in vacuo* before being desalted on a preliminary Celite column. The concentrated neutral Dowex 50 eluate (about 10ml. of solution) was adjusted to pH below 2 with 50% (w/v) H_2SO_4, and 50ml. of butan-1-ol was added with stirring, followed by 10g. of Celite and 50ml. of chloroform. This material was transferred to a short column containing 15g. of Celite and 6ml. of 0·2N-H_2SO_4 previously equilibrated with butan-1-ol–chloroform (1:1, v/v). The base and cap packed to form a column 16cm.×2·8cm. diam. Extraction of the mixed acids was performed with 400ml. of butan-1-ol–chloroform (1:1, v/v) and the acids were re-extracted into dilute NaOH after addition of a further 250ml. of chloroform. The combined extracts and washings, after neutralization with H_2SO_4, were evaporated just to dryness *in vacuo* and dissolved in 1ml. of 3N-H_2SO_4 for final column chromatography as described above. With this procedure the recoveries obtained of 60μmoles of organic acids in 210ml. of incubation medium were: propionic acid, 89%; acetic acid, 96%; fumaric acid, 98·2%; lactic acid plus methylmalonic acid (not resolved), 100·2%; succinic acid, 100·1%; malonic acid, 97·5%; L-malic acid, 98·8%; citric acid plus isocitric acid (partially resolved), 97·2%. The method did not resolve *cis*-aconitic acid and malic acid, nor a mixture of α-oxoglutaric acid, malonic acid and *trans*-aconitic acid.

In addition to estimation of the molar quantities of the products of propionate metabolism, and the identification by scintillation counting of the labelled substances present, the appropriate column eluate fractions were retained for determination of the specific activities of succinic acid, fumaric acid, malic acid, citric acid and propionic acid. After removal of solvents *in vacuo* succinic acid was treated with acid permanganate (Friedemann & Kendall, 1929) to destroy any lactic acid present. Malic acid was separated from any *cis*-aconitic acid present by chromatography on a Dowex 1 (formate form) column with a gradient of formic acid (Busch, Hurlbert & Potter, 1952). Fumaric acid was not further purified, but recombined with the purified malic acid and succinic acid, and the mixture was rechromatographed as before with titration of the fractions. On the basis of these titrations the eluted acids were diluted 200-fold with authentic carrier acids (similarly standardized), cations removed with Dowex 50 and the acids recrystallized three times without measurable change in specific activity. Specific activity was determined on each set of crystals by scintillation counting, and on the final samples by combustion and counting as $^{14}CO_2$. Succinic acid and fumaric acid were recrystallized from water and malic acid was recrystallized from acetone–benzene. Citric acid was not purified before the addition of carrier and was contaminated with an unknown acidic substance that was eluted at this position on the first column. This substance did not react as citric acid by the colorimetric method of Saffran & Denstedt (1948); the carrier dilution of citric acid was based on this method, and the specific activity was determined after recrystallization three times from water. Good agreement was obtained between specific activities of the acids determined in this way and those determined by direct measurement of radioactivity off the first column after suitable corrections for titration blanks in the unincubated flasks. This finding constitutes evidence for the positive identification of the radioactive products detected on the first column. Propionic acid was diluted with carrier after steam-distillation and titration of a sample of the propionic acid eluted from the column. The specific activity was determined by combustion of the silver salt and counting of $^{14}CO_2$.

Consumption of propionic acid was determined by steam-distillation and titration of four replicate samples of the protein-free supernatants of each of the initial and incubated flasks.

The initial samples precipitated after 19min. incubation without added substrate did not contain measurable quantities of succinic acid, fumaric acid, malic acid or citric acid (less than 1μmole). In addition to the unknown acid eluted at the citric acid position, both incubated and initial flasks contained an unknown acid that preceded and partly overlapped the elution of malic acid.

Degradation of labelled products. Distribution of ^{14}C in propionic acid and succinic acid was determined after Schmidt degradation by the method of Phares (1951), the final methylamine or ethylenediamine being oxidized to $^{14}CO_2$ by persulphate (Abraham & Hassid, 1957). Fumaric acid was reduced to succinic acid with zinc and H_3PO_4 in the presence of $CuSO_4$ (Krebs, Smyth & Evans, 1940). The succinic acid was recovered with diethyl ether in a liquid/liquid extractor, recrystallized from water and degraded as above. Malic acid was degraded by two separate procedures. C-2 and C-3 of malic acid were obtained by Schmidt degradation of C-1 and C-2 of the acetic acid derived from malic acid (Swim & Utter, 1957). C-1 and C-4 of malic acid were derived separately via coumalic acid as described by Benson & Calvin (1957). In all cases evolved $^{14}CO_2$ was trapped in carbonate-low NaOH and released and counted as described under 'estimation of ^{14}C'.

Estimation of ^{14}C. ^{14}C was estimated either by scintillation counting in the phosphor solution described by Bray (1960), or as $^{14}CO_2$ in the gas phase by the method of Brown & Miller (1947) as described by Smith & Osborne-White (1965). The procedures used for scintillation counting were as follows. (1) Effluent fractions from Celite columns (0·1ml. samples of 10ml. fractions) were each dissolved in 5ml. of Bray's solution and 4ml. was counted. The counting efficiency depended on the effluent solvent and varied from 44·8% for chloroform to 50·3% for chloroform–butan-1-ol (3:2, v/v). (2) Solid samples of recrystallized carriers (5–10mg.) were each dissolved in 5ml. of Bray's solution and 4ml. was counted (efficiency 68·1%). (3) For the estimation of specific activity of propionic acid or of residual

^{14}C in propionic acid in flask contents after incubation (Expt. 3), samples containing 4–10 μmoles of [2-^{14}C]propionate were distilled at pH2 in the presence of $MgSO_4$, 100ml. of distillate being collected. Then 50ml. of the distillate was titrated and replicate 0·5ml. samples of the residual 50ml. were dissolved in 5ml. of Bray's solution and 4ml. was counted (efficiency 70·3%). As a routine, scintillation counting was for 1000sec. or 10000counts. The equipment described by Smith *et al.* (1965*b*) was used.

Counting of $^{14}CO_2$ trapped in NaOH was performed after release of the $^{14}CO_2$ into a stream of O_2 (by adding excess of 2N-H_2SO_4 at 0°) and trapping in a liquid-N_2 trap (Smith & Osborne-White, 1965). The sample of $^{14}CO_2$ was separated as before from water, metered with a mercury manometer, diluted with unlabelled CO_2 to the appropriate pressure and counted in the presence of CS_2 as before (efficiency 52·4%). Combustion of samples to $^{14}CO_2$ and counting in the gas phase was as described by Smith & Osborne-White (1965).

The counting efficiencies reported are based on a sample of [1,4-$^{14}C_2$]succinic acid, obtained from The Radiochemical Centre, diluted to contain 1mμc of ^{14}C/mg. of succinic acid.

Consumption of propionate. This was determined by steam-distillation and titration of residual volatile acid in flask contents after incubation as described by Smith & Osborne-White (1965). Initial values were determined in flask contents to which protein precipitant was added at the time of addition of substrate.

Nitrogen. Nitrogen in homogenates and mitochondria was determined by the Kjeldahl procedure of McKenzie & Wallace (1954).

RESULTS

Formation of products and recovery of ^{14}C in aged nuclear-free homogenate metabolizing [2-^{14}C]propionate (Expt. 1). The products recovered after incubation of 35ml. of nuclear-free homogenate of sheep liver with 1050 μmoles of [2-^{14}C]propionate are shown in Table 1, together with the specific activities of substrate and products. The homogenate contributed 129mg. of N and was derived from 5g. of liver. Substrate was added after 19min. incubation, when negligible quantities of the intermediates listed were present. The values given therefore represent net formation from propionate. The results are the means ± S.E.M. of duplicate incubations.

In addition to the dicarboxylic acids previously detected as products of propionate metabolism (Smith & Osborne-White, 1965), some citrate accumulated. The molar recovery of propionate in these products (assigning 2mol. of propionate/mol. of citrate) is 63·9%, leaving 36% not accounted for. Assuming this to have been completely oxidized, the extent to which propionate was oxidized was 44%.

The recovery of consumed ^{14}C in all listed products, however, was only 33%. The specific activities of the products, particularly citrate, were considerably less than those expected from the substrate, and only 1·4% of the consumed label appeared as respiratory $^{14}CO_2$. A portion of the missing label was recovered from the Dowex 50 column used for the initial removal of cations, by elution with 2 bed vol. of 3N-hydrochloric acid and 2 bed vol. of water. On evaporation to dryness and extraction of the residue with Bray's (1960) solution a mean of 143mμc of ^{14}C was recovered. This was subsequently identified by paper chromatography with butan-1-ol–water–propionic acid (91:64:45, by vol.) on Whatman no. 1 paper (Edwards, Gadsden, Carter & Edwards, 1959) and detection of ^{14}C by strip-scanning (Smith & Osborne-White, 1965) as labelled glutamate and aspartate, chiefly the latter. This represents only 15% of the missing label, but is a minimum estimate since neither

Table 1. *Products of [2-^{14}C]propionate metabolism by aged nuclear-free homogenate (Expt. 1)*

Nuclear-free homogenate (129mg. of N) derived from 5g. of sheep liver was incubated at 37° for 19min. without added substrate, [2-^{14}C]propionate (5mM) was added and incubation was continued for 36min. Consumption of propionate and production of the intermediates listed were determined as described in the Materials and Methods section. Apart from labelled glutamate and asparate, these were the only radioactive products detected. Control incubations stopped after the preliminary 19min. were devoid of detectable quantities of dicarboxylic acids and citrate. Incubations were performed in 210ml. of the medium described, with a gas phase of O_2+CO_2 (95:5). Results are the means ± S.E.M. of duplicate incubations.

Substrate or product	Quantity consumed or produced (μmoles)	Specific activity (mμc/μmole)	Radioactivity consumed or incorporated (mμc)
Propionate	−72±8	19·08±0·18	−1374
Succinate	+13·4±1·2	12·32±0·18	+165
Fumarate	+5·0±0·3	9·15±0·33	+46
Malate	+18·4±2·7	9·86±0·08	+181
Citrate	+4·6±3·0	8·6±2·1	+40
CO_2	—	0·00245±0·00013	+19
Total products			+451

Table 2. *Distribution of* ^{14}C *in* [2-^{14}C]*propionate and the succinate produced after incubation with aged nuclear-free homogenate* (*Expt.* 1)

The results refer to the same experiment as Table 1(which shows the specific activities). Distribution of label was determined after isolation as described in the Materials and Methods section. There was no change in specific activity or distribution of label in propionate on incubation. Results are the means of triplicate degradations on duplicate incubated samples.

	% of label in carbon atoms			
Substance	C-1	C-2	C-3	C-4
Propionate	0·0	99·8	0·2	—
Succinate	2·3	47·7	47·7	2·3

complete recovery by the Dowex column nor complete extraction into Bray's (1960) solution was ensured.

Measurement by scintillation counting of ^{14}C in the Celite-column fractions revealed no other radioactive products. No label appeared in the small amount of acetic acid detected, and no labelling of lactate occurred, although about 90 μmoles of lactate were present both immediately before and after incubation with labelled propionate. The absence of label from the lactate peak also indicated the absence of any labelled methylmalonic acid free for extraction by the procedure used.

The distribution of label in the propionate and succinate from this experiment is given in Table 2. Both the specific activity and the distribution of label in propionate were identical before and after incubation, the latter values being recorded. The finding of a small proportion of label in C-1 and C-4 of succinate indicates some complete cycling of succinate (2mol. of succinate + 3·5mol. of oxygen $\rightarrow$1mol. of succinate).

The low specific activities of the products, together with the appearance of label in glutamate and aspartate, are consistent with extensive exchange of label between labelled oxaloacetate and α-oxoglutarate and endogenous aspartate and glutamate. Both glutamate and aspartate are known to be present in sheep-liver homogenates (Smith *et al.* 1965*a*).

Formation of products and recovery of ^{14}C *in aged mitochondria metabolizing* [2-^{14}C]*propionate* (*Expt.* 2). This experiment was performed similarly except that washed mitochondria (83mg. of N) derived from 15g. of liver were used in each flask.

Recovery of products and specific activities are shown in Table 3, which also includes the radioactivity found in glutamate and aspartate recovered from the initial Dowex 50 column and counted under standardized conditions. Some radioactivity was also recovered in the elution position of α-oxoglutaric acid (malonic acid and *trans*-aconitic acid are also eluted at this position), and this is also shown in Table 3. The molar quantities shown for the main products represent net formation, as there were negligible quantities of these intermediates present after the preliminary 19min. incubation. The results are similar to those of nuclear-free homogenate, except that proportionately more malate and particularly citrate accumulated. The specific activities of the products approached much more nearly that of the substrate. The recovery of some labelled α-oxoglutarate and the appearance of about

Table 3. *Products of* [2-^{14}C]*propionate metabolism by aged mitochondria* (*Expt.* 2)

Twice-washed mitochondria (83mg. of N) derived from 15g. of sheep liver were incubated at 37° for 19min. without added substrate, [2-^{14}C]propionate (5mM) was added and incubation was continued for 36min. Consumption of propionate and production of the intermediates listed were determined as described in the Materials and Methods section. No other labelled products were detected. Control incubations stopped after the preliminary 19min. incubation were devoid of detectable quantities of dicarboxylic acids and citrate. Incubations were performed in 210ml. of the medium described with a gas phase of $O_2 + CO_2$ (95:5). Results are the means $\pm$ S.E.M. of duplicate incubations.

Substrate or product	Quantity consumed or produced (μmoles)	Specific activity (mμc/μmole)	Radioactivity consumed or incorporated (mμc)
Propionate	−93 ± 10	17·5 ± 0·6	−1628
Succinate	+14·1 ± 0·5	15·8 ± 0·5	+223
Fumarate	+6·7 ± 0·6	14·6 ± 1·2	+98
Malate	+28·7 ± 0·4	15·9 ± 0·3	+456
Citrate	+16·0 ± 0·9	29·5 ± 0·2	+472
CO_2	—	0·014 ± 0·001	+109
α-Oxoglutarate	—	—	+60
Glutamate + aspartate	—	—	+65
Total products	—	—	+1483

Table 4. *Distribution of* ^{14}C *in* [2-^{14}C]*propionate and some of its products after incubation with aged mitochondria* (*Expt.* 2)

The results refer to the same experiment as Table 3 (which shows the specific activities). Distribution of label was determined as described in the Materials and Methods section. There was no change in the specific activity or distribution of label in propionate on incubation. Results are the means of duplicate degradations on duplicate incubated samples.

	% of label in carbon atom			
Substance	C-1	C-2	C-3	C-4
Propionate	0·0	99·9	0·1	—
Succinate	5·4	44·6	44·6	5·4
Fumarate	3·6	46·4	46·4	3·6
Malate	4·0	47·1	45·3	3·6

7% of the consumed ^{14}C in respiratory carbon dioxide probably reflect a much lower extent of exchange of label with endogenous amino acids in the absence of the soluble fraction of the homogenate.

In this experiment 88% of the propionate consumed was accounted for on a molar basis in dicarboxylic acids and citric acid. Assuming the residual 12% to have been completely oxidized the total extent of oxidation of propionate was 30%. The recovery of ^{14}C in all labelled products was 91% of the [2-^{14}C]propionate consumed.

Distribution of label in those products degraded is given in Table 4. As with nuclear-free homogenate there was no loss in specific activity or randomization of label on incubating [2-^{14}C]-propionate, but more extensive labelling of the carboxyl groups of succinate occurred, and similar results were obtained with fumarate. The degradation of malate in which all four carbon atoms were obtained separately showed that the molecule was labelled symmetrically, consistent with its formation via succinate and fumarate.

Although citrate was not degraded, its specific activity was entirely consistent with its having been formed from the malate present via 2 mol. of oxaloacetate, one of which was decarboxylated first to pyruvate, then to acetyl-CoA. The observed specific activity of citrate was 96% of that predicted from this sequence.

The experimental results are consistent with the metabolism of propionate having occurred solely by way of the methylmalonate pathway to succinate followed by oxidation of succinate via pyruvate and the tricarboxylic acid cycle. No other labelled products were detected, but the consumed ^{14}C was still not entirely accounted for and a further experiment was performed in an attempt to account quantitatively for the ^{14}C consumed.

Recovery of ^{14}C *in the products of* [2-^{14}C]*propionate metabolism by aged mitochondria* (*Expt.* 3). Conditions of incubation and the preparation and estimation of samples are described in the Materials and Methods section. Recovery of ^{14}C in intermediates was determined by measuring the specific activity of recovered carriers that were added in large excess (500 mg.) of the amounts of intermediates that could have been produced (0·82 mg. of propionate consumed).

Table 5 shows the consumption and recovery of ^{14}C in the intermediates listed. The total recovered radioactivity in the substances listed did not differ significantly at the 5% level (*t* test) from the ^{14}C consumed as propionate. The total radioactivity in products other than succinic acid, fumaric acid, malic acid, citric acid and the respiratory carbon dioxide was less than 6% of the ^{14}C consumed, of which oxaloacetic acid and aspartic acid accounted for almost 4%.

Table 5. *Distribution of label among the products of* [2-^{14}C]*propionate metabolism by aged mitochondria* (*Expt.* 3)

The [2-^{14}C]propionate consumed was $11·2 \pm 0·2$ μmoles with specific activity 195 mμc/μmole or 2165 ± 39 mμc (mean of six replicates). Replicate flasks containing mitochondria with 1·93 mg. of N were incubated for 19 min. at 37° without added substrate, [2-^{14}C]propionate (5 mM) was added and incubation was continued for 36 min. in 6 ml. final volumes of the medium described, with a gas phase of $O_2 + CO_2$ (95:5). At the conclusion of incubation CO_2 in some flasks was trapped in alkali and each of the remaining substances listed was recovered in excess of carrier and recrystallized to constant specific activity to determine total incorporation of ^{14}C. The procedures employed are described in the Materials and Methods section. The results are the means $\pm$ S.E.M. of duplicate incubations for each product, except for CO_2 which is the mean of four replicates. The estimate of total ^{14}C in products did not differ significantly (5% level by the *t* test) from the total ^{14}C consumed as [2-^{14}C]propionate.

Product	^{14}C recovered (mμc)	
Malate	817 ± 98	(2)
Succinate	808 ± 53	(2)
Citrate	410 ± 15	(2)
Fumarate	80 ± 20	(2)
CO_2	49 ± 8	(4)
Oxaloacetate	46	(2)
Aspartate	37 ± 3	(2)
Methylmalonate	14 ± 1	(2)
Pyruvate	10	(2)
Glutamate	10	(2)
α-Oxoglutarate	7	(2)
Total products	2290 ± 115	

DISCUSSION

Previous indications that propionate metabolism in sheep-liver homogenates proceeded by the methylmalonate pathway through succinate were given by the complete dependence of propionate metabolism on carbon dioxide, the accumulation of dicarboxylic acids as the sole detected products and the adequacy of the activities of enzymes of the methylmalonate pathway to account for the rates of propionate consumption found with nuclear-free homogenates or slices (Smith & Osborne-White, 1965). The present work indicates that, in mitochondria that have been aged for 19min. at 37° without substrate, propionate metabolism took place entirely via succinate, with limited oxidation by way of pyruvate and the tricarboxylic acid cycle. The intermediates of this pathway together with glutamate, aspartate and respiratory carbon dioxide accounted entirely for the ^{14}C consumed as [2-^{14}C]propionate, and the specific activities of the products together with the distribution of label within the molecules were entirely consistent with the suggested sequence. In particular the symmetrical labelling of malate suggests that it was formed from succinate and not by way of the alternative pathway via oxaloacetate suggested by Willmer & Gutfreund (1963) to operate in guinea-pig mammary-gland slices. The results for nuclear-free homogenate, though not so clear-cut in that complete recovery of ^{14}C was not obtained, are nevertheless consistent with the pathway suggested together with extensive exchange of label with endogenous amino acids. The absence of any labelling of endogenously produced lactate supports the previous conclusion (Smith & Osborne-White, 1965) that in aged nuclear-free homogenates lactate is not involved in propionate metabolism.

The difference in stability to aging of nuclear-free homogenates and mitochondria in their capacity to metabolize propionate is reflected in the present results in the lower rates (per g. of original liver) obtained with mitochondria; the rates with nuclear-free homogenate were 2·3-fold higher. This was accompanied by the accumulation of a greater proportion of the metabolized propionate as malate, and particularly as citrate, in the case of mitochondria. Gallagher & Buttery (1959) showed that citrate, malate and fumarate were more readily oxidized by whole sucrose homogenates of sheep liver than by isolated mitochondria. It therefore appears probable that one of the effects of the soluble fraction of the homogenate was to permit more complete oxidation of the accumulated citrate and malate. This may have contributed to the higher rate of propionate metabolism by formation of stimulatory amounts of α-oxoglutarate, although both citrate and isocitrate were just as effective as α-oxoglutarate in stimulating the rate of propionate metabolism in aged mitochondria, and the major stimulatory effect of the soluble fraction undoubtedly arose from the glutamate present (Smith *et al.* 1965*a*,*b*).

Very little accumulation of ^{14}C occurred in keto acids in Expt. 3, oxaloacetate containing more than either pyruvate or α-oxoglutarate. Since pyruvate is known to stimulate propionate metabolism in aged mitochondria, and oxaloacetate to inhibit it (Smith *et al.* 1965*b*), a limitation in the rate of propionate metabolism may have been imposed by the rate of conversion of oxaloacetate into pyruvate in the mitochondria. Considerable amounts of pyruvate (17% of the propionate consumed) must, however, have been formed to account for the citrate that accumulated.

The extent of oxidation of propionate obtained with nuclear-free homogenate (44%) is consistent with the correlation between the rates of consumption of oxygen and propionate previously found and equivalent to 50% oxidation of the propionate consumed (Smith & Osborne-White, 1965), since some oxidation of endogenous glutamate to aspartate undoubtedly occurred (Smith *et al.* 1965*b*). However, it is clear that this extent of oxidation was not essential for propionate metabolism to occur in mitochondria, where the extent of oxidation was only 30%. The efficiency of energy production and utilization was thus lower in nuclear-free homogenate than in mitochondria, and so it cannot be postulated that the higher rate of the nuclear-free homogenate was due to the higher rate of oxygen consumption as such.

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