Metabolism of Propionate by Sheep Liver

OXIDATION OF PROPIONATE BY HOMOGENATES

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1. The rate and stability to aging of the metabolism of propionate by sheep-liver slices and sucrose homogenates were examined. Aging for up to 20 min. at 37° in the absence of added substrate had little effect with slices, whole homogenates or homogenates without the nuclear fraction. 2. Metabolism of propionate by sucrose homogenates was confined to the mitochondrial fraction, but the mitochondrial supernatant (microsomes plus cell sap) stimulated propionate removal. 3. The rate of propionate metabolism by liver slices was higher in a high potassium phosphate-bicarbonate medium [$0.88 (\pm \text{s.e.m. } 0.16) \mu\text{mole/mg. of N/hr.}$] than in Krebs-Ringer bicarbonate medium [$0.44 (\pm \text{s.e.m. } 0.13) \mu\text{mole/mg. of N/hr.}$]. 4. Metabolism of propionate by sucrose homogenates freed from nuclei was dependent on the presence of oxygen, carbon dioxide and ATP. Propionate removal was stimulated 250% by Mg^{2+} ions and 670% by cytochrome *c*. 5. In the complete medium $2.39 (\pm \text{s.e.m. } 0.15) \mu\text{moles}$ of propionate were consumed/mg. of N/hr. 6. The ratio of oxygen consumption to propionate utilization was sufficient to account for the complete oxidation of half the propionate consumed. 7. The only products detected under these conditions were succinate, fumarate and malate. Propionate had no effect on the production of lactate from endogenous sources and did not itself give rise to lactate. 8. Methylmalonate did not accumulate when propionate was metabolized and was not oxidized. It was detected as an intermediate in the conversion of propionyl-CoA into succinate. The rate of this reaction sequence was adequate to account for the rate of propionate metabolism by sucrose homogenates or slices, provided that the rate of formation of propionyl-CoA was not limiting. 9. The methylmalonate pathway was predominantly a mitochondrial function. 10. The metabolism of propionate appeared to be dependent on active oxidative phosphorylation.

During an investigation of the metabolic defect occurring in vitamin B₁₂ deficiency in sheep (Marston, Allen & Smith, 1961) a study was made of the metabolism of propionate by homogenates of normal sheep liver. The present series of papers describes these findings and their subsequent extension. A preliminary note of part of the present work has appeared (Smith & Osborne-White, 1961).

Short-chain fatty acids produced during ruminal fermentation of plant materials constitute in large part the energy source of ruminants (Marston, 1948). The particular importance of propionate as a source of glucose in ruminants was pointed out by Phillipson (1947). The ruminant absorbs negligible quantities of free glucose from the intestinal tract and relies on gluconeogenesis, chiefly from propionate, as a means of maintaining the carbohydrate

reserve of the body. Short-chain fatty acids absorbed from the rumen are carried by the portal blood to the liver before entering the general circulation. Annison, Hill & Lewis (1957), by examination of arterial and portal blood in sheep, obtained evidence indicating that propionate is very largely removed from the blood in its passage through the liver.

In the present investigation the metabolism of propionate has been examined in sheep-liver slices and in homogenates of sheep liver prepared in 0.25 M-sucrose.

MATERIALS AND METHODS

Animals. Australian Merino ewes, aged 2–5 years, were removed from pasture 1 week before slaughter, housed in pens and fed *ad lib.* on a diet of 50% of wheaten hay chaff

and 50% of dried lucerne chaff (by wt.). All animals were allowed access to food up to the time of slaughter, and were killed by cutting the throat.

Homogenates. For manometric work, precooled pieces of the livers of freshly killed animals were extruded through circles of 16-mesh stainless-steel gauze supported in a syringe barrel. A 1:7 homogenate (1.0 g. of liver mash plus 6.0 ml. of 0.25 M-sucrose) was prepared at 0° with a Potter-Elvehjem homogenizer (Potter & Elvehjem, 1936). The homogenate was centrifuged for 10 min. at 600g in an International refrigerated centrifuge, and the supernatant fluid that was employed for manometric work contained an average of 3.5 mg. of N/ml. Microscopic examination after vital staining with Janus Green B showed that the 600g supernatant, comprising a suspension of mitochondria and microsomes, was essentially free from cell fragments and nuclei, and that the 600g sediment consisted mostly of unbroken cells together with cell fragments, nuclei (clearly visible as unstained voids), erythrocytes and some sedimented mitochondria.

In large-scale experiments one part of liver to five parts of 0.25 M-sucrose was used with a homogenizer of 150 ml. capacity fitted with a polyethylene pestle manufactured as described by Kamphausen & Morton (1956). The nuclear-free homogenate (600g supernatant) in these experiments contained an average of 4.3 mg. of N/ml., and (Table 3) about 31% of the nitrogen of the whole homogenate, and 16% of the total activity with propionate was rejected in the 600g sediment. Centrifugal fractionation of homogenates was performed by the method of Hogeboom, Schneider & Pallade (1948).

The temperature was maintained between 0° and 3° throughout the preparation.

Disrupted mitochondria (Tables 6 and 7) were prepared by homogenization of whole liver or isolated mitochondria for 3.5 min. at top speed at 0° in a Servall Omnimixer (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) in 0.04 M-tris-HCl buffer, pH 8.

Manometric procedure. Rates of consumption of O₂ and evolution of CO₂ were measured in an atmosphere of O₂+CO₂ (95:5) by Warburg's indirect method (Dixon, 1951, p. 76; Umbreit, 1949).

In early experiments with nuclear-free homogenates it was found that reproducible rates could be obtained with propionate as substrate only when stringent precautions were taken to ensure gas saturation of the medium at all stages where homogenate was present. This led to rather prolonged gassing (9 min.) and equilibration (10 min.) stages in the bath, and it was found that, when substrate had been present from zero time, only relatively brief periods (15–20 min.) of respiration at linear rates could be observed. It was then found that if propionate was added at the conclusion of the gassing and equilibration times (i.e. after 19 min. in the bath, this period being determined in part by manipulative considerations, in handling two banks of six flasks) linear rates of consumption of O₂ could be measured for the final 30 min. of a 36 min. incubation. This procedure was adopted, but, since it involved incubation of the nuclear-free homogenate for 19 min. in the absence of substrate before measurement of respiration, a study was made of the stability of various centrifugal fractions of the homogenate to such incubation. Table 2 shows that only a small fraction of the capacity to metabolize propionate is lost during 19 min. incubation both in the

whole homogenate (average loss 8%) and in the nuclear-free homogenate (average loss 5%). Table 1 shows that such stability to incubation in the absence of substrate is also found in sheep-liver slices metabolizing propionate in Krebs–Ringer bicarbonate medium.

The standard medium in which gas exchange and consumption of substrate was measured had the following final composition: K₂HPO₄ (16.4 mm), KH₂PO₄ (3.6 mm), NaHCO₃ (25.0 mm), KCl (90 mm), MgCl₂ (5.0 mm), sucrose (46.3 mm), ATP (1.3 mm), cytochrome *c* (11 μm), substrate (5.0 mm; added after 19 min. in the bath) and liver protein contributing 2.2–5.5 mg. of N to a final volume of 6.0 ml. in equilibrium at 37° with O₂+CO₂ (95:5) (final pH 7.4).

The medium was kept saturated with gas mixture at room temperature before and during addition of the nuclear-free homogenate. After a 9 min. gassing period in the bath flasks were equilibrated for 10 min. and substrate in buffer was added from the side arm. Gas exchange was measured in pairs of flasks at 6 min. intervals for 36 min., the first reading taken 6 min. after adding substrate being taken as the zero point of the measurement. The values quoted for rates of consumption of O₂ thus represent the period from 6 to 36 min. after the addition of substrate; rates for consumption of substrate were measured over the entire incubation period.

Measurements of gas exchange were made in conventional Warburg flasks (20–25 ml. volume) in final volumes of 6.0 and 3.0 ml. (or 2.0 ml.) containing 1.0 and 0.5 ml. (or 0.33 ml.) of the nuclear-free homogenate respectively. (Flasks containing 6.0 ml. volumes were without centre wells.) Shaking rates of 130–140 7 cm.-oscillation cycles/min. were employed, and measured respiration was proportional to the amount of nuclear-free homogenate. Measurements of consumption of O₂ with propionate as substrate were regarded as valid only when it had been shown that the rates of consumption of substrate (per ml. of nuclear-free homogenate) were identical in the two flasks constituting a measurement.

Under these conditions replicate measurements of propionate oxidation indicated errors (95% confidence interval of the mean of three replicates) of ±6% for O₂ and ±9% for CO₂. The validity of the method of measurement was tested by a study of the oxidative decarboxylation of *p*-hydroxyphenylpyruvate by the enzyme *p*-hydroxyphenylpyruvate hydroxylase (EC 1.99.1.14), prepared from pig liver by the method of Hager, Greggerman & Knox (1957). Satisfactory agreement with the direct method for O₂ was obtained.

Rates quoted represent the total exchanges of O₂ measured in pairs of flasks. No corrections have been applied for gas exchange in the absence of substrate, although these rates were measured. The conditions of the experiments were chosen to obtain linear rates of consumption of O₂ over the 30 min. period from 6 to 36 min. after adding substrate. Rates in the absence of substrate, however, were generally not linear, but fell off with time.

Incubation of homogenate fractions without manometric measurements. In the experiments listed in Tables 2 and 3, where multiple measurements of propionate consumption by homogenate fractions were made, and in the experiment described in Table 4, where measurements were made in three different gas phases, centrifugal fractions of sucrose homogenates were incubated in the reaction vessels and

under the conditions described by Smith, Osborne-White & Russell (1965). Reaction tubes in these experiments were supported in racks to permit simultaneous incubation of up to 50 tubes. The only difference in the conditions of these experiments from those performed in Warburg flasks was that all vessels were continually flushed with gas mixture (200 ml./min. to each flask) throughout the entire incubation. Similar precautions with regard to the initial saturation of the medium with gas mixture were employed as in experiments where gas exchange was measured.

Reactions were terminated by the addition of 1 ml. of 10% (w/v) sodium tungstate dihydrate and 1 ml. of 0.6*N*-H₂SO₄. Water was added to give a volume of 20 ml. and the tube contents were centrifuged to remove protein. Consumption of volatile acid was estimated by difference from initial values determined at the time of tipping. Values quoted are the means of duplicate incubations.

Preparation and incubation of liver slices. Liver slices were cut and incubation begun within 1 hr. of killing the animals. Plugs of liver for preparation of slices were cut from the liver immediately after death, with a steel cork borer of 1.0 cm.² cross-section, and were plunged into ice-cold 0.25*M*-sucrose to chill. After draining, slices (0.4–0.6 mm. thick) were cut by hand in a cold room at 0–1° by the method of Deutsch (1936), weighed and placed directly in 5.4 ml. of pre-gassed medium at 0–1°. Four slices (total weight about 200 mg.) were added to each vessel. Vessels were flushed continuously with O₂+CO₂ (95:5) gas mixture both during the preparation of slices and throughout the incubation period. The reaction vessels and the method of incubation were as described by Smith *et al.* (1965).

After the stated period of incubation at 37° in the absence of substrate, buffered substrate (0.6 ml. containing 30 μmoles of potassium propionate) was added from the side arm and incubated for 36 min. with shaking. Reactions were terminated by the addition of 2 ml. of 2*N*-H₂SO₄, water was added to give a volume of 20 ml. and the vessel contents were centrifuged to remove slices and fragments.

Residual volatile acid was determined in samples of the supernatant, and consumption determined from control flasks to which acid was added at the time of tipping. All values quoted for consumption of propionate represent the means of duplicate incubations.

Two media were employed to measure slice metabolism: Krebs–Ringer bicarbonate medium (Cohen, 1949), and the standard medium employed for measurement of homogenate metabolism, but from which ATP and cytochrome *c* were omitted.

Incubation of Omnimixer homogenates with ¹⁴CO₂ and isolation of products. Animals were killed and sections of liver homogenized in a Servall Omnimixer at 0–2° in 9 vol. of 0.04*M*-tris-HCl buffer, pH 8.0, containing GSH (1 mm), for 3.5 min. at top speed. For enzyme assay two dilutions of the homogenate were prepared in 0.04*M*-tris-HCl buffer, pH 7.4, containing GSH (1 mm).

Incubations were carried out in short tubes (internal diam. 15 mm.) of total volume about 6 ml., sealed with rubber serum caps. The composition of the medium was as follows: tris-HCl buffer, pH 7.4 (50 mm), GSH (5 mm), MgCl₂ (3 mm), ATP (3 mm), propionyl-CoA (1 mm), Na₂¹⁴CO₃ (10 mm; specific activity 200 mμC/μmole) and liver homogenate contributing 0.425–3.16 mg. of protein to a final

volume of 1 ml. Incubation in a gas phase of air was for 20 min. at 30°, and reactions were started by the addition of Na₂¹⁴CO₃ by syringe through the serum cap.

The reactions were terminated by the addition of 0.2 ml. of 2*N*-KOH, and tubes were allowed to stand for 1 hr. at room temperature to hydrolyse CoA thio esters. Carrier acids (125 μg. each of methylmalonic acid, succinic acid, fumaric acid, malic acid, malonic acid and citric acid) were then added, the solution was acidified with 0.5 ml. of 4*N*-HCl and ¹⁴CO₂ was discharged in a fume hood for 1 hr. Residual ¹⁴CO₂ was removed by leaving the tubes overnight in an evacuated desiccator over 2*N*-NaOH. Tubes were then heated for 1 min. in a boiling-water bath and the contents transferred quantitatively in 0.1*N*-HCl to tared centrifuged tubes and weighed (final volume about 8 ml.). Tubes were centrifuged to remove protein, and, after removal of small weighed samples (about 0.2 g.) for determination of total fixation of ¹⁴CO₂ by combustion, most of the remainder was extracted for 24 hr. with freshly distilled peroxide-free diethyl ether in a liquid-liquid extractor.

The ether was evaporated on a rotary evaporator, and the residue taken up in water and transferred with washing to 15 ml. conical centrifuge tubes. These solutions were evaporated to dryness by heating in a water bath (80°) under a stream of N₂. The residue was taken up in a small volume of water (0.2 ml.), and samples (50 μl.) were spotted on Whatman no. 1 paper and chromatographed for 17 hr. at 25° in 3-methylbutan-1-ol saturated with 4*N*-formic acid (Flavin & Ochoa, 1957).

After spraying lightly with bromophenol blue, radioactive spots were located with a strip scanner, cut out and burnt to ¹⁴CO₂ for determination of ¹⁴C as described below. Recovery of ¹⁴C by combustion of paper as compared with the initial determination of total fixation was 95%.

Incubation of Omnimixer homogenates with [2-¹⁴C]-propionyl-CoA and isolation of products. A homogenate (1:10) in 0.25*M*-sucrose was prepared and the mitochondria were isolated and washed twice as described above. Portions of the mitochondria were then suspended in equal volumes of 0.08*M*-tris-HCl buffer, pH 8.0, containing GSH (2 mm), and either 0.25*M*-sucrose or the mitochondrial supernatant, and homogenized for 3.5 min. at top speed in a Servall Omnimixer at 0–2°. A further portion of the mitochondrial supernatant was mixed with an equal volume of the same buffer and similarly homogenized for 3.5 min.

Samples (0.4 ml.) of the three homogenates were incubated with [2-¹⁴C]propionyl-CoA in a medium of the following final composition: [2-¹⁴C]propionyl-CoA (1 mm; specific activity 330 mμC/μmole), tris-HCl buffer, pH 7.5 (50 mm), MgCl₂ (3 mm), MnCl₂ (1.3 mm), NADP (0.3 mm), GSH (5 mm) and where appropriate NaHCO₃ (3 mm) and ATP (3 mm) in final volumes of 1 ml.

Incubations in a gas phase of air were carried out for 20 min. at 30° in short tubes (internal diam. 15 mm.) of about 6 ml. volume, sealed with rubber serum caps. The reaction was started by the addition of NaHCO₃ by syringe through the serum cap and terminated by the addition of 1 drop of 8*N*-NaOH (final pH > 12). Flasks were then allowed to stand for 1 hr. at room temperature to hydrolyse CoA thio esters.

Carrier acids (125 μg. each of methylmalonic acid, succinic acid, fumaric acid, malic acid, malonic acid and citric acid) were added in 0.1 ml. and tube contents acidified (pH < 2) with 1 drop of 50% (v/v) H₂SO₄. After standing

for 1 hr. in a fume hood tubes were placed overnight in an evacuated desiccator over 2N-NaOH to remove $^{14}\text{CO}_2$.

The acidified mixture was mixed intimately with 2g. of dry Celite (Celite 545; Johns-Manville Co., New York, N.Y., U.S.A.) suspended in chloroform and applied as a cap to a column of Celite. The column (1cm. diam.), consisted of 5g. of Celite containing 2ml. of 0.2N- H_2SO_4 and was equilibrated with chloroform saturated with 0.2N- H_2SO_4 . The last traces of the reaction mixture in Celite were transferred to the column with the aid of glass wool moistened with chloroform and the cap was packed tightly.

Radioactive propionate was eluted from the column in 125 ml. of chloroform previously saturated with 0.2N- H_2SO_4 . The mixed carrier acids were then eluted with 150 ml. of butan-1-ol-chloroform (1:1, v/v) previously saturated with 0.2N- H_2SO_4 . This procedure was based on the method of Swim & Krampitz (1954) for the chromatographic separation of organic acids and was found to achieve a complete separation of propionic acid from the non-volatile carrier acids listed above.

The eluted carrier acids were extracted from the organic solvent into an excess of NaOH (5 ml. of 2N) with the aid of a vibrating emulsifier, and the aqueous phase was recovered after centrifugation. The organic phase was washed twice in a similar manner with 2 ml. of 0.02N-NaOH, and the aqueous phases were combined. The aqueous solution containing the carrier acids was then acidified (pH < 2) with H_2SO_4 and extracted for 24 hr. with freshly distilled peroxide-free diethyl ether in a liquid-liquid extractor.

The ether was evaporated, the residue transferred quantitatively with ether to a 15 ml. centrifuge tube and the ether again evaporated. The residue was concentrated in the tip of the tube and dissolved in 0.2 ml. of water. Samples (50 μl .) of this solution were spotted on Whatman no. 1 paper and chromatographed for 17 hr. at 25° in 3-methylbutan-1-ol saturated with 4N-formic acid. After being dried, papers were sprayed and radioactive spots detected with a strip scanner. Spots were cut out and burnt to CO_2 to determine ^{14}C .

Incubation of nuclear-free homogenates with propionate and CoA, and detection of hydroxamic acids. The medium was similar to the standard medium, but contained a higher concentration of ATP (2.6 mM) and where appropriate additional CoA (1 mM) and neutralized hydroxylamine. Final volumes of 2 ml. were employed containing 0.5 ml. of nuclear-free homogenate (2.07 mg. of N) and potassium propionate (5 mM). All components except the nuclear-free homogenate were added to the main chamber and pregassed at room temperature with $\text{N}_2 + \text{CO}_2$ (95:5). Reactions were started by the addition of the nuclear-free homogenate and the flasks were attached to manometers, placed in the bath and regassed for 9 min. with shaking. After a further 28 min. neutralized hydroxylamine (final concn. 0.67 M or 1 M) or tungstic acid was added to terminate the reactions.

After standing at room temperature for 15 min. the contents of the flasks containing hydroxylamine were added to 25 vol. of ethanol and centrifuged to remove protein. The ethanol extract was evaporated to dryness at room temperature. The crystalline residue was triturated three times with 0.7 ml. portions of ethanol and filtered through small plugs of glass wool in capillary tubes. The combined filtrates were evaporated to dryness at room

temperature under a stream of N_2 , taken up in 0.2 ml. of ethanol, and 50 μl . samples were spotted on Whatman no. 1 paper with and without standard acetyl-, propionyl- and succinyl-hydroxamic acid. The standards were prepared from acid anhydrides by the method of Lipmann & Tuttle (1945).

Chromatograms were developed with butan-1-ol-acetic acid-water (4:1:5, by vol.), as described by Thompson (1951), and sprayed with the FeCl_3 reagent described by Stadtman & Barker (1950).

Materials. The NaHCO_3 (A.R.) was obtained from Standard Laboratories (Pty) Ltd., Melbourne, Victoria, Australia; K_2HPO_4 (pure grade) was obtained from Hopkin and Williams (Pty) Ltd., Chadwell Heath, Essex. 2,4-Dinitrophenol, propionic anhydride, malonic acid, succinic acid and fumaric acid were laboratory-reagent-grade chemicals obtained from British Drug Houses Ltd., Poole, Dorset; succinic acid, fumaric acid and malonic acid were recrystallized from water and dried before use. L-Malic acid, GSH and crystalline bovine albumin were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Disodium ATP (from muscle), CoA and NADP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Propionic acid (laboratory-reagent grade) was obtained from British Drug Houses Ltd. and fractionally distilled twice. The final material was collected over the boiling range 141–142.5° and was demonstrated to be free (less than 1%) of homologous short-chain fatty acids by the gas-liquid chromatographic method of James & Martin (1952).

Other chemicals were of analytical-reagent grade. Solvents were redistilled.

Cytochrome *c* was prepared from horse heart by the method of Keilin & Hartree (1945) or purchased from the Sigma Chemical Co. (horse heart, type III) and was estimated by the spectrophotometric method of Potter (1949).

Methylmalonic acid was prepared by saponification, extraction into ether after acidification, and recrystallization of a sample of the diethyl ester obtained through the courtesy of Dr J. A. Mills of this Laboratory. The ester had been prepared from diethyl malonate and methyl iodide by the method of Weiner (1943).

Propionyl-CoA was prepared from propionic anhydride and CoA by the method of Simon & Shemin (1953). Excess of anhydride and free propionic acid were removed after acidification with HCl by extraction with three successive lots of 3 vol. of diethyl ether. The solution was then brought to pH 5 with NaHCO_3 and stored at –20°. Propionyl-CoA was estimated by the method of Grunert & Phillips (1949).

[2- ^{14}C]Propionyl-CoA was prepared from sodium [2- ^{14}C]propionate, obtained from The Radiochemical Centre, Amersham, Bucks. The sodium [2- ^{14}C]propionate (100 μC in 23.5 μmoles) was diluted with a solution of unlabelled sodium propionate to a specific activity of 0.33 $\mu\text{C}/\mu\text{mole}$. The water was evaporated and the residue dried in a small glass-stoppered reaction tube at 190° for 2 hr. in a stream of dry N_2 . The material was ground with a glass rod and redried before conversion into the anhydride with sulphur and bromine by a semi-micro modification of the method of Orshansky & Bograchov (1944). The thoroughly ground and dried sodium [2- ^{14}C]propionate (300 μmoles) was mixed by means of a glass bead with 6 μl . (17.7 mg.; density 2.945) of a solution of 0.272 g. of resublimed sulphur in

2.99 g. of pure bromine. The tube was stoppered and sealed heavily with wax before heating at 50° with continuous mechanical vibration for 2 hr. The propionic anhydride was distilled by the 'cold-finger' technique under high vacuum (10⁻⁵ mm. Hg) with liquid N₂. The yield of anhydride was 69% as estimated by the hydroxamic acid method of Lipmann & Tuttle (1945). The anhydride was then converted into [2-¹⁴C]propionyl-CoA as described above, and estimated as thiol group appearing on alkaline hydrolysis. The overall yield from [2-¹⁴C]propionate was 23%.

The Na₂¹⁴CO₃ was obtained from The Radiochemical Centre and diluted with unlabelled Na₂CO₃ to a specific activity of 0.2 μC/μmole.

Chemical methods. Reactions in Warburg flasks were terminated by the addition of 1 ml. of 10% (w/v) sodium tungstate dihydrate, and 1 ml. of 0.66 N-H₂SO₄/6 ml. of flask contents. After dilution with water to 20 ml. and centrifugation to remove protein, the supernatant was frozen in glass-stoppered containers before assay. In experiments involving the detection of ether-soluble products, tungstic acid was replaced with Zn(OH)₂ as protein precipitant. In this case 6 ml. of flask contents was treated with 0.5 ml. of 25% (w/v) ZnSO₄·7H₂O and equivalent NaOH (phenolphthalein end point) in 1.5 ml. This treatment eliminated contamination of the supernatant with ether-soluble lipids, but allowed complete recovery of propionic acid, estimated as described below.

Volatile acid in protein-free supernatants was estimated after steam-distillation by titration under N₂. Samples containing 4–10 μmoles of propionic acid were distilled at constant volume from 20 ml. of 50% (w/v) MgSO₄·7H₂O adjusted to pH 2 (Congo red paper) with H₂SO₄. Distillate (100 ml.) was collected over about 40 min. and 50 ml. samples were titrated. The distillation flask was fitted with an efficient Cyclone-type centrifugal splash head designed by Mr V. A. Stephen of this Laboratory. Distillation vapour entered the lagged 7 cm.-diam. hollow glass disk tangentially and left through a take-off normal to the centre of the disk. Centrifugal force in the rapidly spinning vapour stream prevented the carry over of spray droplets to the take-off. With selected samples of MgSO₄, blank titrations of 3–4 μl. of 0.01 N-NaOH/50 ml. of distillate were regularly obtained.

Titration were performed with 0.01 N-NaOH (carbonate-free) delivered from an Agla micrometer syringe. Titration vessels were treated with silicone (Desicote) and were loosely covered during titration. Nitrogen (freed from CO₂ and NH₃) was bubbled constantly through the solution during titration and for 3 min. beforehand. The course of the titration was followed with a glass electrode and a Cambridge portable pH-meter, the end point being taken as pH 7.0.

Recovery of acetic acid and propionic acid was within ±3% over the range 4–10 μmoles distilled.

In experiments where manometric measurements were made, consumption of propionate over 36 min. was estimated by difference from an initial value comprising substrate plus nuclear-free homogenate. The nuclear-free homogenates contained approx. 1 μmole of volatile acid/ml., chiefly acetic acid, and this was not significantly depleted during the 55 min. incubation period either in the presence or absence of 30 μmoles of propionate. Apart from the disappearance of a trace (less than 0.2 μmole) of formic acid

present in the homogenate, consumption of propionic acid was the only change detected.

In experiments that did not involve manometric measurements the consumption of volatile acid was measured by difference from initial values determined at the time of tipping. In either case values quoted for rates of consumption of volatile acid represent the means of at least two replicate incubations.

Lactate was estimated by the colorimetric procedure of Barker & Summerson (1941) after copper-lime treatment of samples of the protein-free supernatant. Crystalline zinc lactate was employed as reference standard.

Total nitrogen in homogenate fractions and slices was determined by the Kjeldahl procedure of McKenzie & Wallace (1954).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine albumin as reference standard.

Paper chromatography. Flask contents were treated with Zn(OH)₂ to remove protein and 0.75 samples evaporated just to dryness under reduced pressure. The residue was taken up in 2 ml. of water, acidified with H₂SO₄ (pH < 2) and mixed with 25 g. of anhydrous NaSO₄. The acids were extracted in a Soxhlet apparatus for 6 hr. with freshly distilled peroxide-free diethyl ether and the ether was evaporated. After redissolving in known volumes of water samples were spotted on Whatman no. 1 paper with and without authentic acids and chromatographed. The four systems employed were: *A*, ethanol-aq. NH₃ (sp. gr. 0.88)-water (8:1:1, by vol.), as described by Jones, Dowling & Skrabala (1953); *B*, diethyl ether-acetic acid-water (13:3:1, by vol.) on Whatman no. 1 paper washed with 1% aq. NH₃ and dried (Denison & Phares (1952)); *C*, 3-methylbutan-1-ol saturated with 4N-formic acid (Flavin & Ochoa, 1957); *D*, ethyl methyl ketone-water-acetone-formic acid (40:6:3:1, by vol.), as described by Högström (1957).

Estimation of ¹⁴C. The ¹⁴C was estimated as ¹⁴CO₂ by the method of Brown & Miller (1947), by the vacuum techniques described by Glascock (1954a). The dimensions of the combustion tube were increased to permit combustion of spots cut from paper chromatograms. The total length of the quartz combustion tube (internal diam. 10–12 mm.) was 125 cm. The length of the catalytic filling ('simple band' filling of Niederl & Niederl, 1942) was 45.6 cm., maintained at 700° with an electric furnace. Samples were burnt at 800° with a second electric furnace preceding the catalytic filling and combustion gases were trapped in liquid N₂ in the multiple U trap described by Buchanan & Nakao (1952).

After metering (mercury manometer) ¹⁴CO₂ was supplemented with unlabelled CO₂ to give a total pressure in the counting tube of 200 mm. Hg and with CS₂ to give a partial pressure of 20 mm. Hg and counted. Counting tubes of the type described by Glascock (1954b) with a tungsten anode and stainless-steel cathode were obtained from 20th Century Electronics Ltd., New Addington, Surrey (type GA10/M). An external quenching circuit (Neher & Harper, 1936) was employed and the operating potential was 3760 v. Commercial power supply and scaling equipment were used. Counting efficiency was 52.4% (counts as percentage of disintegrations in the tube), and recovery of carbon was 100 ± 0.5% from a range of compounds including stearic acid and glutamic acid. Recovery of carbon from

spots cut from paper chromatograms was quantitative up to 70 mg./paper. Overall errors in the estimation of ^{14}C based on replicate measurements over several years were $\pm 2\%$ when 10^4 counts were recorded.

In experiments involving the recovery of labelled compounds from paper chromatograms by combustion of the paper, papers were first sprayed lightly with indicator to locate carrier acid spots, and location of ^{14}C was determined with a strip-scanning device by employing a thin end-window Geiger-Müller tube (General Electric Co. model E.H.M.2). Radioactive areas were cut out with scissors, allowing about 0.25 in. beyond the visible spot, and burnt in quartz boats.

EXPERIMENTAL AND RESULTS

Rate of metabolism of propionate by sheep-liver slices and stability to preliminary incubation in the absence of substrate. The rate of metabolism of propionate by sheep-liver slices was determined in five experiments with two different media (Tables 1 and 2). When substrate was added within 2 min. of placing in the bath the rate in Krebs-Ringer bicarbonate medium was $0.44 (\pm \text{s.e.m. } 0.13) \mu\text{mole/mg. of N/hr.}$, and in the standard medium (with ATP and cytochrome *c* omitted) was $0.88 (\pm \text{s.e.m. } 0.16) \mu\text{mole/mg. of N/hr.}$

The results in Table 1 were subjected to analysis of variance. The analysis showed that in Krebs-Ringer bicarbonate medium there was no significant change in activity ($P > 0.05$) on incubating slices

for up to 60 min. in the absence of substrate. In the standard medium the rate fell on incubation in the absence of substrate, but the fall over the first 20 min. was not significant ($P > 0.05$), and in two further experiments (Table 2) no loss in activity was observed on incubating slices in standard medium for 19 min. in the absence of substrate. The rate in the standard medium was significantly higher ($P < 0.001$) than in Krebs-Ringer bicarbonate medium when substrate was present at the beginning of the incubation.

Sheep-liver slices thus exhibit considerable stability to preliminary incubation in their capacity to metabolize propionate under the conditions employed.

Location of activity in liver homogenates and stability to incubation in the absence of substrate. Table 3 shows the mean activity of the various centrifugal fractions expressed as a percentage of mean activity in the whole homogenates and compared with the mean distribution of nitrogen in the fractions. The mitochondrial supernatant itself was entirely inactive, the fraction metabolizing propionate at the highest rate (per ml.) being the 600g supernatant. The mitochondria had the highest specific activity in terms of nitrogen.

Study of the stability of the fractions to a preliminary incubation of 19 min. in the absence of substrate (Table 2) showed that very little loss of activity occurred during this period either with the whole homogenate (average loss 8%) or with the 600g supernatant (average loss 5%; see also Table 4).

Marked loss of activity (average 46%) occurred on incubating the isolated mitochondria in the absence of substrate, but this could be restored to a considerable extent by the addition of the mitochondrial supernatant after the preliminary incubation of 19 min. Under these conditions the mean loss of activity due to incubation of the mitochondria was decreased to 25%. This loss on aging of the intrinsic capacity of mitochondria to metabolize propionate was surprisingly small.

From the results it appeared that the effect of the mitochondrial supernatant was twofold; it exerted a marked stimulating effect on the rate of metabolism of propionate by the mitochondria, and it protected the mitochondria from inactivation during the 19 min. period in the absence of substrate.

The 600g sediment, consisting of unbroken cells, cell fragments, nuclei, erythrocytes and some mitochondria, contained 16% of the activity of the homogenate but about 30% of the nitrogen. This fraction was discarded in the experiments described below, which were performed on the 600g supernatant (nuclear-free homogenate).

Dependence of propionate metabolism on carbon dioxide and oxygen and stimulation by Mg^{2+} ions.

Table 1. *Rate of metabolism of propionate by sheep-liver slices and stability to incubation in the absence of substrate*

Conditions of the experiments are described in the Materials and Methods section. The standard medium was that employed for homogenate metabolism but with ATP and cytochrome *c* omitted. The gas phase was $\text{O}_2 + \text{CO}_2$ (95:5), and propionate consumption was determined over 36 min. after incubation of the slices in the absence of substrate for the stated period. Initial volatile acid was determined in control flasks to which acid was added at the time of tipping. The results are the means $\pm$ s.e.m. of three separate experiments. In each experiment duplicate vessels were incubated and the mean value of the propionate consumption was used for the calculation.

Time of preliminary incubation at 37° without substrate (min.)	Propionate consumed ($\mu\text{moles/mg. of N/hr.}$)	
	Krebs-Ringer bicarbonate medium	Standard medium
0	0.60 ± 0.15	1.08 ± 0.19
10	0.63 ± 0.17	1.01 ± 0.07
20	0.65 ± 0.13	0.89 ± 0.02
40	0.62 ± 0.06	0.74 ± 0.05
60	0.72 ± 0.08	0.64 ± 0.15

Table 2. *Rate of metabolism of propionate by sheep-liver slices and centrifugal fractions of sucrose homogenates measured before and after aging in the absence of substrate*

Slices were incubated either in Krebs-Ringer bicarbonate medium or in the standard medium with ATP and cytochrome *c* omitted. Homogenates (1:6) were prepared in 0.25M-sucrose and centrifugally fractionated as described in the Materials and Methods section. The 600g sediment was resuspended on 0.25M-sucrose and made to the original whole-homogenate volume. The mitochondria were washed twice and resuspended in 0.25M-sucrose to the volume of nuclear-free homogenate from which they were derived. The reconstituted nuclear-free homogenate was prepared by resuspending twice-washed mitochondria in the mitochondrial supernatant to the original volume of nuclear-free homogenate from which they were derived. All homogenate incubations were performed in the standard medium, and all incubations took place for 36 min. at 37° after the addition of substrate in a gas phase of O₂+CO₂ (95:5). Initial volatile acid was determined at the time of the addition of substrate. Each value quoted is the mean of duplicate incubations.

Preparation	Expt. no.	N content (mg./ml. of homogenate fraction or mg./g. of slices)	Time of preliminary incubation without substrate at 37° ...	Propionate consumed (μmoles/mg. of N/hr.)	
				2 min.	19 min.
Slices in Krebs-Ringer bicarbonate medium	1	30.2		0.26	0.36
	2	29.4		0.16	0.27
Slices in standard medium (no ATP or cytochrome <i>c</i>)	1	30.2		0.56	0.59
	2	29.4		0.57	0.61
Whole sucrose homogenate	1	5.35		2.55	2.27
	2	5.10		2.95	2.79
600g Sediment in original homogenate volume	1	1.69		1.28	1.08
	2	1.51		1.68	1.00
600g Supernatant (nuclear-free homogenate)	1	4.42		3.31	2.94
	2	4.19		2.76	2.82
Twice-washed mitochondria	1	1.02		4.75	2.94
	2	0.88		6.03	2.79
Mitochondrial supernatant (microsomes+ cell sap)	1	3.22		Nil	Nil
	2	3.22		Nil	Nil
Reconstituted nuclear-free homogenate	1	3.96		2.95	2.44
	2	3.93		3.04	2.78
Mitochondria incubated for 19 min. in absence of substrate before adding mitochondrial supernatant	1	3.96		—	2.19
	2	3.93		—	2.39

Table 3. *Distribution of propionate-metabolizing activity in centrifugal fractions of sucrose homogenates of sheep liver*

The results of Expts. 1 and 2 (Table 2) are expressed as mean distribution of total nitrogen and total activity. The results are derived from the values in Table 2 for activity determined after 2 min. incubation without substrate, and from measurements of the volumes recovered in the various fractions.

Homogenate fraction	Percentage of total N	Percentage of total activity
Whole homogenate	100	100
600g Sediment	30.6	16.4
600g Supernatant (nuclear-free homogenate)	69.4	75.0
Mitochondria	15.3	29.7
Mitochondrial supernatant (microsomes+ cell sap)	51.9	Nil

Table 4 shows the complete dependence of propionate metabolism in the nuclear-free homogenate on the presence of carbon dioxide (or bicarbonate) and on oxygen. Also shown is the marked stimulation in rate due to Mg²⁺ ions, determined both after 2 and 19 min. incubation in the absence of substrate.

Dependence on ATP and the effect of dinitrophenol. The dependence of propionate metabolism in the nuclear-free homogenate on the presence of ATP is shown in Table 5. Substrate was added after 19 min. incubation at 37°. The effect of ATP reached a maximum at about 1 mM.

In a further experiment the effect on consumption of propionate of 30 μM-dinitrophenol was determined. Duplicate pairs of flasks were incubated containing in final volumes of 6 ml. of standard medium 30 μmoles of propionate (added after 19 min.) and 1 ml. of nuclear-free homogenate

Table 4. *Dependence of propionate metabolism in the nuclear-free homogenate on oxygen and carbon dioxide and stimulation by Mg^{2+} ions*

Homogenate (1:6) of sheep liver was prepared in 0.25 M-sucrose and a nuclear-free homogenate prepared (4.21 mg. of N/ml.). After the stated period at 37° in the absence of substrate, propionate was added from the side arm and incubated for 36 min. in standard medium with $O_2 + CO_2$ (95:5) (complete), standard medium with $N_2 + CO_2$ (95:5) (no O_2), standard medium with $NaHCO_3$ omitted and gas phase 100% O_2 (no CO_2), or standard medium with $MgCl_2$ omitted and gas phase $O_2 + CO_2$ (95:5) (no Mg^{2+} ions). Initial volatile acid was determined at the time of tipping. Values for uptakes represent the means of triplicate incubations, except in the complete system for which both values are the means of six replicate incubations.

System	Time of preliminary incubation without substrate at 37° ... 2 min.	Propionate consumed (μ moles/mg. of N/hr.)	
		19 min.	
Complete		2.79	2.62
No O_2		Nil	—
No CO_2		Nil	—
No Mg^{2+} ions		0.19	0.48

Table 5. *Dependence of propionate metabolism on ATP in the presence of oxygen + carbon dioxide (95:5)*

The conditions of the experiment and the composition of the medium (excepting ATP) were as described in the Materials and Methods section. Concentrations of ATP from 0 to 1.5 mM were present in the main chamber in the presence of nuclear-free homogenate during the gassing and equilibration period of 19 min. at 37° before the addition of propionate from the side arm to give a final concentration of 5 mM. Consumption of propionate was measured over 36 min., as described in the Materials and Methods section. Final volumes of 6 ml. containing 1.0 ml. of nuclear-free homogenate (3.11 mg. of N) were employed, and gas phase was $O_2 + CO_2$ (95:5).

Concn. of added ATP (mM)	Propionate consumed (μ moles/mg. of N/hr.)
0	0.0
0.5	1.2
1.0	2.4
1.5	2.6

containing 4.13 mg. of N. After 36 min. incubation with substrate flasks without dinitrophenol had consumed 5.0 and 5.1 μ moles of propionate, whereas no consumption was detected in flasks containing dinitrophenol.

From the above results and from the dependence on oxygen it may be inferred that propionate metabolism in these preparations was dependent

on the process of oxidative phosphorylation. The presence of ATP may also have served to protect the mitochondria against the aging process.

Stimulation by cytochrome c. Added cytochrome c stimulated the rate of consumption of propionate and oxygen by the nuclear-free homogenate. The endogenous respiration was not increased by additional cytochrome c. The maximum rate both of uptake of oxygen and consumption of propionate was reached with 5 μ M-cytochrome c.

Rate of metabolism of propionate and acetate. In a series of 18 experiments the rate of consumption of propionate by nuclear-free homogenates of sheep liver was measured in the standard medium after 19 min. incubation in the absence of substrate. The mean rate of consumption of propionate was 2.39 ($\pm$ S.E.M. 0.15) μ equiv./mg. of N/hr. The values obtained lay within the range 1.6–3.2 μ equiv./mg. of N/hr.

In a series of three experiments under identical conditions, no consumption of acetate was detected either in the presence or absence of 0.67 mM-L-malate. In the same experiments propionate was metabolized at rates within the normal range.

Relationship of oxygen consumption to propionate utilization. In 14 measurements with nuclear-free homogenates a mean of 1.74 ($\pm$ S.E.M. 0.04) μ moles of oxygen were consumed for each μ mole of propionate disappearing. The correlation between total oxygen and propionate is approximately equivalent to the complete oxidation of half the propionate metabolized.

Production of lactate. Leng & Annison (1963) with sheep-liver slices, and Pennington & Sutherland (1956) with slices of sheep rumen epithelium, found lactate to be a major end product of propionate metabolism. In sheep-liver nuclear-free homogenates, added propionate (final concn. 5 mM) had no effect on the rate of lactate production (eight observations), and thus the two processes of metabolism of propionate and production of lactate appeared to proceed independently.

Production of ether-soluble non-volatile acids. Ether-soluble acids produced during the metabolism of propionate by nuclear-free homogenates were detected by paper chromatography in four different solvent systems as described in the Materials and Methods section. Satisfactory resolution of a mixture of malate, lactate, succinate, methylmalonate and fumarate was achieved, and the presence of malate, succinate and fumarate demonstrated. No methylmalonate was formed. The acids were not detected unless propionate was added as substrate. From an assessment based on spot sizes and limits of detection it was estimated that a total of 2.7 μ moles of succinate, fumarate and malate had been produced during the loss of 4.7 μ moles of propionate.

Evidence for the formation of propionyl-CoA. Metabolism of propionate to succinate and L-malate is consistent with the operation in sheep liver of the methylmalonate pathway of propionate metabolism (Mazumder, Sasakawa, Kaziro & Ochoa, 1961). The first reaction in the sequence is the formation of propionyl-CoA, presumably by means of the acetate-activating enzyme reported by Hele (1954). Accordingly, attempts were made to detect the formation of propionyl-CoA in the nuclear-free homogenate incubated with propionate and CoA. Formation of CoA thio esters was detected after treatment with hydroxylamine, essentially by the method of Stadtman & Barker (1950). All flasks containing both propionate and CoA showed a well-defined spot chromatographically identical with propionylhydroxamic acid. Flasks containing propionate only showed no spots.

The formation of the material identified in this way with propionylhydroxamic acid was thus dependent both on propionate and on CoA. The amounts formed, however, were small and were not visibly increased by the inclusion of hydroxylamine in flask contents during incubation at 37°.

Identification of methylmalonate as an intermediate. Flavin, Ortiz & Ochoa (1955) observed that rat-liver homogenates oxidized methylmalonate in the presence of bicarbonate at about two-thirds the rate at which propionate was oxidized. In the present study it was found in two experiments that methylmalonate (5mM) was without effect on the respiration of sheep-liver nuclear-free homogenates under conditions where propionate was rapidly oxidized. This finding, together with the failure to detect methylmalonate among the products of propionate metabolism, led to a closer examination of the initial steps in the metabolism of propionate by sheep-liver homogenates.

The conditions employed to allow the formation

of methylmalonate to be detected were similar to those used by Flavin, Castro-Mendoza & Ochoa (1957) in studying carboxylating-enzyme activity in pig-heart preparations. Whole homogenates of sheep liver were prepared in a high-speed blender in 0.04M-tris-hydrochloric acid buffer, pH 8, to disrupt mitochondrial structure, and fixation of $^{14}\text{CO}_2$ from $\text{NaH}^{14}\text{CO}_3$ was measured in the presence of propionyl-CoA. After alkaline hydrolysis of CoA thio esters and extraction into ether, radioactive products were chromatographed with carrier on paper, and the separated products burnt to determine total activity as $^{14}\text{CO}_2$ (Table 6).

Total radioactivity fixed into products was directly related to the amount of homogenate protein, although the rate declined with increasing protein. By taking as the true rate 43.2 $\mu\text{C}/\text{mg.}$ of protein/hr. and applying the specific activity of the substrate $^{14}\text{CO}_2$ (200 $\mu\text{C}/\mu\text{mole}$), the rate of fixation of $^{14}\text{CO}_2$, and so of propionyl-CoA, was 0.216 $\mu\text{mole}/\text{mg.}$ of protein/hr. Assuming that 6.6mg. of protein was equivalent to 1mg. of N (a value obtained by assay of whole liver), then this rate becomes 1.4 $\mu\text{moles}/\text{mg.}$ of N/hr. at 30°. This compares satisfactorily with the mean rate of 2.4 $\mu\text{moles}/\text{mg.}$ of N/hr. obtained with nuclear-free homogenates at 37°, assuming that the rate of formation of propionyl-CoA was not limiting.

The order in which the products began to accumulate with increasing protein is consistent with a reaction sequence in which methylmalonate, succinate and malate were formed in that order.

Intracellular location of the reaction sequence of the methylmalonate pathway. An experiment was performed to detect the distribution between mitochondria and cytoplasm of the overall enzymic activity responsible for the conversion of propionyl-CoA into methylmalonate, succinate and malate.

Table 6. *Radioactive products formed on incubating $\text{NaH}^{14}\text{CO}_3$ and propionyl-CoA with sheep-liver homogenate*

A homogenate of sheep liver was prepared in 0.04M-tris-HCl buffer, pH 8, in a high-speed blender to disrupt mitochondrial structure. Samples of the homogenate were incubated with propionyl-CoA (1mM), GSH (5mM), MgCl_2 (3mM), ATP (3mM) and $\text{NaH}^{14}\text{CO}_3$ (10mM; 200 $\mu\text{C}/\mu\text{mole}$) in tris-HCl buffer, pH 7.4, for 20 min. at 30°. After hydrolysis of CoA thio esters, the ether-soluble products were separated by paper chromatography and burnt before measurement of radioactivity as $^{14}\text{CO}_2$ in the gas phase. Experimental details are given in the text and in the Materials and Methods section.

Protein (mg.)	Separated products ($\mu\text{C}/\text{hr.}$)				Sum of separated products	
	Methylmalonate	Succinate	Fumarate	Malate	Total ($\mu\text{C}/\text{hr.}$)	Rate ($\mu\text{C}/\text{mg.}$ of protein/hr.)
0.425	15.5	1.8	0.8	0.3	18.3	43.2
0.85	18.6	13.7	1.5	1.3	35.0	41.4
1.58	26.3	29.1	2.6	5.4	63.4	40.2
3.16	25.5	51.0	5.4	19.1	101.0	32.1

Mitochondria were isolated, washed twice with 0.25M-sucrose and disrupted in 0.04M-tris-hydrochloric acid buffer, pH 8, by high-speed blending both in the presence and absence of the mitochondrial supernatant (microsomes plus cell sap). Samples of these homogenates and of the mitochondrial supernatant were then incubated with [2-¹⁴C]propionyl-CoA in the presence and absence of bicarbonate and ATP.

The three homogenates were referred to as: disrupted mitochondria (4.12mg. of protein/ml.), mitochondria disrupted in mitochondrial supernatant (10.15mg. of protein/ml.) and mitochondrial supernatant (7.20mg. of protein/ml.). The two mitochondrial preparations contained equivalent concentrations of mitochondria, which were enriched approximately twofold with respect to the concentration of the mitochondrial supernatant.

After paper chromatography in the presence of carrier the radioactive products detected by the strip scanner were: methylmalonate (R_F 0.76), succinate (R_F 0.66), fumarate (R_F 0.81), malate (R_F 0.35), traces of citrate (R_F 0.22) and malonate (R_F 0.54) and considerable activity (up to 10% of the total ¹⁴C recovered) in an unidentified product (R_F 0.86) that travelled ahead of fumarate.

This material (X2) was partially resolved from a second unidentified component (X1, R_F 0.82) that was itself poorly resolved from fumarate. Component X1 was present in all flasks after incubation but may have been a contaminant of the [2-¹⁴C]-propionic acid. The unknown component X2 was formed in quantity from propionyl-CoA only when ATP was present. The three substances, fumarate, X1 and X2, were not sufficiently well resolved for separate determination of ¹⁴C and so have been estimated together. In flasks where the radioactivity in the unknown substances was greater than 3m μ C (9m μ C/hr.), however, more than half of this was attributable to component X2, as judged by the strip-scanner records.

The results of the experiment are given in Table 7. It is clear that the mitochondria were responsible for most of the formation of dicarboxylic acids observed, although some conversion occurred with the mitochondrial supernatant, and a small stimulation was apparent when the two components were incubated together. The unknown product X2, which in flasks containing ATP may be assessed as half the total of X1 + X2 + fumarate, was also dependent for its formation on the mitochondrial fraction. From the strip-scanner records the formation of component X2 was clearly dependent on ATP.

By applying the specific activity of the substrate propionyl-CoA (330m μ C/ μ mole) to the rate of formation of radioactive succinate, malate and methylmalonate (80m μ C/hr. by mitochondria with

Table 7. Radioactive products formed on incubating [2-¹⁴C]propionyl-CoA with disrupted mitochondria in the presence and absence of the mitochondrial supernatant

Mitochondria from sheep liver were isolated in 0.25 M-sucrose and disrupted in a high-speed blender in the presence and absence of the mitochondrial supernatant (microsomes plus cell sap) as described in the text. Samples of the homogenates and of the supernatant alone were incubated for 20 min. at 30° in the presence of [2-¹⁴C]propionyl-CoA and either NaHCO₃ or NaHCO₃ + ATP. The composition of the medium and the conditions of the experiment are described in the text and in the Materials and Methods section. After alkaline hydrolysis of CoA thio esters, the products of the reaction were separated from radioactive propionate, resolved chromatographically, and radioactivity (as ¹⁴CO₂) was determined as described in the text and in the Materials and Methods section.

	Radioactive products formed (mμC/hr.)									
	Addition ...	Disrupted mitochondria (1.65 mg. of protein)			Mitochondrial supernatant (2.88 mg. of protein)			Mitochondria disrupted in mitochondrial supernatant (4.06 mg. of protein)		
		None	NaHCO ₃	NaHCO ₃ + ATP	None	NaHCO ₃	NaHCO ₃ + ATP	None	NaHCO ₃	NaHCO ₃ + ATP
Malate	—	—	14.1	—	—	0.2	—	—	23.2	
Succinate	0.9	0.9	40.2	1.1	1.2	2.6	2.7	2.5	50.1	
Methylmalonate	1.1	0.4	25.2	1.1	1.2	7.5	1.3	1.2	33.1	
Sum of malate, succinate and methylmalonate	2.0	1.3	79.5	2.2	2.4	10.3	4.0	3.7	106.4	
Unknown products X1 + X2, plus fumarate	5.1	4.4	17.4	3.5	3.9	6.7	5.9	2.6	16.4	

Mitochondria from sheep liver were isolated in 0.25M-sucrose and disrupted in a high-speed blender in the presence and absence of the mitochondrial supernatant (microsomes plus cell sap) as described in the text. Samples of the homogenates and of the supernatant alone were incubated for 20 min. at 30° in the presence of [2-¹⁴C]propionyl-CoA and either NaHCO₃ or NaHCO₃ + ATP. The composition of the medium and the conditions of the experiment are described in the text and in the Materials and Methods section. After alkaline hydrolysis of CoA thio esters, the products of the reaction were separated from radioactive propionate, resolved chromatographically, and radioactivity (as ¹⁴CO₂) was determined as described in the text and in the Materials and Methods section.

1.65 mg. of protein), and converting mg. of mitochondrial protein into mg. of N (6.6 mg. of mitochondrial protein = 1 mg. of N), a rate of formation of dicarboxylic acids of about $1 \mu\text{mole/mg. of N/hr.}$ at 30° is derived. This value is well below the rate of metabolism of propionate by mitochondria of about $5.4 \mu\text{moles/mg. of N/hr.}$ at 37° in the standard medium when substrate was added at 2 min. (Table 2). The difference between this rate and the rate obtained from the results in Table 6 is unexplained but may represent one or more of several differences in the medium; in particular, the concentration of bicarbonate was lower in the experiment referred to in Table 7.

The experiment serves to demonstrate the existence of an ATP-dependent formation of methylmalonate, succinate and malate from propionyl-CoA in mitochondria.

DISCUSSION

Leng & Annison (1963), working with sheep-liver slices, found that incubation with labelled propionate caused introduction of label into glucose. The pattern of labelling was consistent with the formation of glucose from propionate via a symmetrical intermediate such as succinate. No net synthesis of glucose occurred, however, and lactate was the major product. Lactate was also the sole product detected by Pennington & Sutherland (1956) after metabolism of propionate by slices of sheep-rumen epithelium. In the presence of malonate, however, radioactive propionate gave rise to succinate of high specific activity, and Pennington & Sutherland (1956) concluded that metabolism of propionate occurred via fixation of carbon dioxide to form succinate.

With the exception of a doubt as to the rate of formation of propionyl-CoA, the results of the present paper are entirely consistent with the metabolism of propionate proceeding in sheep-liver homogenates via a mitochondrial methylmalonate pathway (Mazumder *et al.* 1961; Mazumder, Sasakawa & Ochoa, 1963). The failure to detect methylmalonate among the ether-soluble reaction products may be ascribed to the extraordinary stability of the thio ester bond of methylmalonyl-CoA (Stadtman, Overath, Eggerer & Lynen, 1960) leading to a failure to extract the acid into ether. The rate of fixation of $^{14}\text{CO}_2$ by high-speed-blender homogenates in the presence of propionyl-CoA is adequate to account for the rate of metabolism of propionate by sucrose homogenates, which in turn is more than adequate to account for the rate in liver slices.

Nuclear-free homogenates, however, appear to differ from slices in the nature of the products. No lactate whatever was produced by homogenates

from propionate, whereas it appeared to be the sole product in slices of liver or rumen epithelium. The dicarboxylic acids that accumulate in nuclear-free homogenates may represent precursors of the lactate produced by slices.

The correlation between the rate of consumption of oxygen and that of propionate was unexpected. It is consistent with the accumulation of dicarboxylic acids, which was approx. 57% of the propionate consumed on a molar basis. Pennington & Sutherland (1956) found that up to 50% (38–49% in four observations) of metabolized propionate appeared as lactate in slices of rumen epithelium.

The extent to which endogenous substrates contribute to the consumption of oxygen when propionate is present cannot be gauged, and so the fraction of metabolized propionate that is completely oxidized is not known. The correlation between total oxygen and propionate removed, however, suggests that a connexion exists between the rate at which tricarboxylic acid-cycle intermediates are oxidized and the rate of consumption of propionate.

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REFERENCES

- Annison, E. F., Hill, K. J. & Lewis, D. (1957). *Biochem. J.* **66**, 592.
Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Brown, S. C. & Miller, W. M. (1947). *Rev. sci. Instrum.* **18**, 496.
Buchanan, D. L. & Nakao, A. (1952). *J. Amer. chem. Soc.* **74**, 2389.
Cohen, P. P. (1949). In *Manometric Techniques and Tissue Metabolism*, revised ed., p. 119. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
Denison, F. W. & Phares, E. F. (1952). *Analyt. Chem.* **24**, 1628.
Deutsch, W. (1936). *J. Physiol.* **87**, 56 f.
Dixon, M. (1951). *Manometric Methods*, p. 76. Cambridge: University Press.
Flavin, M., Castro-Mendoza, H. & Ochoa, S. (1957). *J. biol. Chem.* **229**, 981.
Flavin, M. & Ochoa, S. (1957). *J. biol. Chem.* **229**, 965.
Flavin, M., Ortiz, P. J. & Ochoa, S. (1955). *Nature, Lond.*, **176**, 823.
Glascock, R. F. (1954a). *Isotopic Gas Analysis for Biochemists*, pp. 10, 83, 102. New York: Academic Press Inc.
Glascock, R. F. (1954b). *Isotopic Gas Analysis for Biochemists*, p. 63. New York: Academic Press Inc.
Grunert, R. R. & Phillips, P. H. (1949). *J. biol. Chem.* **181**, 820.

- Hager, S. E., Gregerman, R. I. & Knox, W. E. (1957). *J. biol. Chem.* **225**, 935.
- Hele, P. (1954). *J. biol. Chem.* **206**, 671.
- Hogeboom, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.
- Högström, G. (1957). *Acta chem. scand.* **11**, 743.
- James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.
- Jones, A. R., Dowling, E. J. & Skrabala, W. J. (1953). *Analyt. Chem.* **25**, 394.
- Kamphausen, H. & Morton, R. K. (1956). *Biochem. J.* **63**, 647.
- Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
- Leng, R. A. & Annison, E. F. (1963). *Biochem. J.* **86**, 319.
- Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McKenzie, H. A. & Wallace, H. S. (1954). *Aust. J. Chem.* **7**, 55.
- Marston, H. R. (1948). *Biochem. J.* **42**, 564.
- Marston, H. R., Allen, S. H. & Smith, R. M. (1961). *Nature, Lond.*, **190**, 1085.
- Mazumder, R., Sasakawa, T., Kaziyo, Y. & Ochoa, S. (1961). *J. biol. Chem.* **236**, PC53.
- Mazumder, R., Sasakawa, T. & Ochoa, S. (1963). *J. biol. Chem.* **238**, 50.
- Neher, H. V. & Harper, W. W. (1936). *Phys. Rev.* **49**, 940.
- Niederl, J. B. & Niederl, V. (1942). *Organic Quantitative Microanalysis*, 2nd ed., p. 107. New York: John Wiley and Sons Inc.
- Orshansky, J. & Bograchov, E. (1944). *Chem. & Ind.* p. 382.
- Pennington, R. J. & Sutherland, T. M. (1956). *Biochem. J.* **63**, 618.
- Phillipson, A. T. (1947). *Nutr. Abstr. Rev.* **17**, 12.
- Potter, V. R. (1949). In *Manometric Techniques and Tissue Metabolism*, revised ed., p. 213. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
- Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
- Simon, E. J. & Shemin, D. (1953). *J. Amer. chem. Soc.* **75**, 2520.
- Smith, R. M. & Osborne-White, W. S. (1961). *Nature, Lond.*, **192**, 868.
- Smith, R. M., Osborne-White, W. S. & Russell, G. R. (1965). *Biochem. J.* **95**, 423.
- Stadtman, E. R. & Barker, H. A. (1950). *J. biol. Chem.* **184**, 769.
- Stadtman, E. R., Overath, P., Eggerer, H. & Lynen, F. (1960). *Biochem. biophys. Res. Commun.* **2**, 1.
- Swim, H. E. & Krampitz, L. O. (1954). *J. Bact.* **67**, 419.
- Thompson, A. R. (1951). *Aust. J. sci. Res. B*, **4**, 180.
- Umbreit, W. W. (1949). In *Manometric Techniques and Tissue Metabolism*, revised ed., p. 30. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
- Weiner, N. (1943). *Org. Synth., Coll. vol.*, **2**, 279.