Metabolism of Propionate by Sheep Liver

STIMULATION OF THE MITOCHONDRIAL RATE BY FACTORS FROM THE CELL SAP

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1. The rate of metabolism of propionate by aged sheep-liver mitochondria in the presence of oxygen + carbon dioxide (95:5) was $5.0 \pm \text{s.e.m. } 0.8$ $\mu\text{moles/mg. of mitochondrial N/hr.}$ 2. When aged in the presence of the mitochondrial supernatant the rate was increased. Mitochondria from 0.33 g. of liver, when combined with the corresponding mitochondrial supernatant from 0.08 g. of liver, metabolized propionate at a rate of $11.4 \pm \text{s.e.m. } 1.2$ $\mu\text{moles/mg. of mitochondrial N/hr.}$ This rate is comparable with rates previously obtained with aged nuclear-free homogenates. 3. Two factors in the mitochondrial supernatant were detected, which when combined reproduced the effect of the fresh supernatant and prevented loss of activity on aging. One of these was non-diffusible and was recovered by fractionation of the dialysed mitochondrial supernatant with ammonium sulphate. The second factor was present in an ultrafiltrate of fresh mitochondrial supernatant and in boiled mitochondrial supernatant; it was isolated and identified as L(+)-glutamate. 4. The effect of the non-diffusible factor was due to protection of the mitochondria from the aging process, whereas glutamate served both in this capacity and as a direct stimulant of propionate metabolism at low concentration.

It was previously shown (Smith & Osborne-White, 1965) that the mitochondria were responsible for the metabolism of propionate by sheep-liver homogenates, but utilization was faster on the addition of mitochondrial supernatant (microsomes plus soluble fraction). In the presence of the mitochondrial supernatant, but without substrate, the metabolism of propionate was almost stable to aging for 19 min. at 37° with a loss of activity of about 6%. Even when the addition of the mitochondrial supernatant was delayed for 19 min. while the mitochondria were being incubated at 37°, 75% of the rate of the nuclear-free homogenate was restored. Two effects of the mitochondrial supernatant were apparent: first, a direct stimulation of the rate of metabolism of propionate by mitochondria (observable without aging), and, secondly, a protective effect of the mitochondrial supernatant against loss of activity by aging.

The present paper describes the isolation of the factors involved in these effects. The microsomal fraction was without effect, the entire effect being confined to the soluble fraction. The stimulation of the rate of propionate metabolism was found to be due to L-glutamate, and a protective effect against aging was exerted both by glutamate and by a non-

diffusible factor recovered as an ammonium sulphate fraction from dialysed mitochondrial supernatant.

MATERIALS AND METHODS

Materials. The materials employed were identical with those described by Smith & Osborne-White (1965). The K_2HPO_4 (extra pure, dry) was obtained from E. Merck, Darmstadt, Germany, and dried at 110° for 48 hr. before use. The $(\text{NH}_4)_2\text{SO}_4$ (AnalaR grade from British Drug Houses Ltd., Poole, Dorset) was recrystallized from aq. 0.2% EDTA, dried and ground before use. L-Glutamic acid was obtained from British Drug Houses Ltd. and recrystallized from water. Dowex 1 anion-exchange resin (8% cross-linked; 100–200 mesh) was obtained in the Cl^- form from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Amberlite IR4-B (OH^- form) was obtained from British Drug Houses Ltd.; the analytical-grade resin was ground overnight in a ball mill (wet) and the 50–100 mesh material recovered. Cytochrome c (from horse heart) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Glass-distilled water was used throughout.

Animals. Australian Merino ewes (aged 2–5 years) were removed from pasture 1 week or more 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.).

Homogenates. Animals were killed by cutting the throat, the livers rapidly removed and approx. 1 g. pieces chilled in ice-cold 0.25 M-sucrose (11. of 0.25 M-sucrose to 100 g. of

liver). The sucrose was decanted in the cold room (1°) and replaced with a similar quantity to complete the chilling. The pieces of liver were then recovered and blotted dry, and a liver mash was prepared by extrusion through stainless-steel gauze, as described by Smith & Osborne-White (1965). Portions (25 g.) of the liver mash were homogenized for 3 min. in 125 ml. portions of ice-cold 0.25 M-sucrose in a 150 ml. glass homogenizer (polyethylene pestle) supported in an ice-water bath in the cold room (1°). The homogenizer consisted of an unground-glass mortar fitted with a polyethylene pestle on a stainless-steel shaft, as described by Kamphausen & Morton (1956). It was driven at 1400 rev./min. by an electric-drill motor (0.25 h.p.) controlled by a variable transformer.

Unbroken cells, nuclei and cell debris were removed by centrifuging for 10 min. at 600 g in an International refrigerated centrifuge. Mitochondria were isolated and washed twice in 0.25 M-sucrose by the method of Hogeboom, Schneider & Pallade (1948) in a Spinco model L preparative ultracentrifuge. The first mitochondrial supernatant was retained.

The mitochondrial pellet was then suspended in 0.25 M-sucrose to one-eighth or one-quarter of the volume of the nuclear-free homogenate from which it was derived, and finally employed after further dilution with either 0.25 M-sucrose, the mitochondrial supernatant, or fractions derived from the mitochondrial supernatant dissolved in 0.25 M-sucrose.

Preparations of the mitochondria from up to 150 g. of liver mash were made in this way, the time between killing an animal and beginning the incubation of mitochondria being 2.5–3 hr. The temperature was maintained at $0-2^{\circ}$ throughout all preparative manipulations.

All final mitochondrial suspensions used in experiments were enriched twofold with respect to mitochondria, i.e. 1 ml. of final suspension contained the mitochondria isolated from 0.33 g. of liver mash.

Incubations. Homogenates were incubated in 6 ml. volumes of a medium of the following final composition: K_2HPO_4 (16.4 mM), KH_2PO_4 (3.6 mM), NaHCO_3 (25.0 mM), KCl (90 mM), MgCl_2 (5.0 mM), sucrose (46.3 mM), cytochrome *c* (11 μM), ATP (1.3 mM), potassium propionate (5.0 mM) added 2 or 19 min. after placing in the bath, and mitochondria from 0.33 g. of liver mash contributing 1.41–2.07 mg. of N. The medium was in equilibrium with $\text{O}_2 + \text{CO}_2$ (95:5) before and throughout the incubation, homogenate being added immediately before placing in the bath. Bath temperature was 37° and flasks were incubated for 2 or 19 min. in the absence of substrate before buffered potassium propionate was added from the side arm and incubation continued for a further 36 min.

In some experiments reactions were carried out in conventional Warburg flasks, as described by Smith & Osborne-White (1965), but employing in all flasks final volumes of 6 ml. In most experiments, however, incubations were performed in batches of 50 tubes. The tubes employed were Pyrex test tubes (2.4 cm. internal diam. and 8.9 cm. long) provided with side arms of 1.0–1.5 ml. capacity situated 3.2 cm. from the bottoms of the tubes. Each tube was fitted with a rubber stopper carrying an inlet tube (0.2 cm. internal diam.) for delivery of gas, and a vent tube (0.6 cm. internal diam.) that served also for introduction of mitochondria.

The tubes were set up in two racks of 25. Each rack consisted of a wooden plank 73 cm. long with tubes clipped on

either side. The top edge of the plank carried a thick-walled white-rubber hose (2 cm. external diam., 0.8 cm. internal diam.) from which projected 25 lengths (4 cm.) of 0.5 mm. (internal diam.) glass capillary tube connected by 12 cm. lengths of rubber tubing to the inlet tubes of the reaction vessels.

The tube racks were set up in the cold room (1°), substrate was added to the side arms, and gas flow was begun at approx. 200 ml. of water-saturated gas mixture [$\text{O}_2 + \text{CO}_2$ (95:5)]/min. to each vessel. Gas flow was begun about 1 hr. before the addition of mitochondria and simultaneously the medium concentrate was gassed by bubbling with gas mixture. Gas flow to each vessel was tested, and after 30 min. 4.4 ml. of the pregassed medium concentrate was added to each vessel. Mitochondria at 0° were then added, tube vents stoppered and racks transferred to the 37° bath, where stoppers were removed and gassing was continued at a similar rate for the remainder of the incubation. During incubation racks were mechanically shaken at 80–100 7 cm. oscillation cycles/min. After 2 or 19 min. at 37° racks were briefly removed while substrate was tipped simultaneously from the side arms into all tubes. The composition of the substrate was such that there was no change in the concentration of buffer, sucrose or KCl on tipping. Incubation in the presence of substrate was continued for 36 min. with constant gassing and shaking.

At the conclusion of incubation racks were removed from the bath and placed in an ice-water bath, and 1 ml. of 10% (w/v) sodium tungstate dihydrate and 1 ml. of 0.66 N- H_2SO_4 were added to each vessel. After the addition of 12 ml. of water, vessel contents were centrifuged to remove protein, and the supernatants decanted and frozen (-20°) in glass containers before assay.

Chemical methods. Residual volatile acid was determined in tungstic acid supernatants, as described by Smith & Osborne-White (1965). Net consumption was determined by difference from initial (triplicate) values determined from control flasks precipitated at the time of tipping, after 2 or 19 min. incubation.

Total nitrogen in homogenates was determined by the Kjeldahl procedure of McKenzie & Wallace (1954).

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

EXPERIMENTAL AND RESULTS

To reproduce the conditions under which measurements of respiration had been made (Smith & Osborne-White, 1965), mitochondria were incubated in the presence and absence of factors from the mitochondrial supernatant for 19 min. at 37° before adding propionate. Consumption of propionate was then measured over 36 min. In this way both stimulating and protective effects were observed and these were subsequently resolved by measurement of propionate consumption with and without aging. Fig. 1 shows the effect of increasing quantities of the mitochondrial supernatant on the rate of consumption of propionate by isolated washed mitochondria from sheep liver. The effect was near maximal with 0.5 ml. of supernatant, representing

Fig. 1. Effect of the mitochondrial supernatant on the rate of metabolism of propionate by aged sheep-liver mitochondria. Mitochondria from 0.33 g. of liver were incubated for 36 min. at 37° with potassium propionate (5 mM) after incubation with graded quantities of the mitochondrial supernatant for 19 min. at 37°. In each case 0.5 ml. of mitochondrial supernatant represents 0.08 g. of liver. Consumption of volatile acid was determined (duplicate assays) in tungstic acid-treated flask contents, as described in the Materials and Methods section. Experimental conditions and the composition of the medium are described in the Materials and Methods section; the gas phase was $O_2 + CO_2$ (95:5). In Expt. 5 (○) mitochondria with 1.55 mg. of N were incubated with mitochondrial supernatant containing 3.59 mg. of N/ml. In Expt. 10 (●) mitochondria with 1.75 mg. of N were incubated with mitochondrial supernatant containing 3.10 mg. of N/ml.

0.08 g. of liver. The mitochondria employed in the experiments were derived from 0.33 g. of liver. In 11 experiments this quantity of mitochondrial supernatant stimulated the rate of consumption of propionate by a mean of 270%. The rate in the absence of the mitochondrial supernatant was 5.0 ($\pm$ S.E.M. 0.8) μ moles of propionate consumed/mg. of mitochondrial N/hr. In the presence of the mitochondrial supernatant the rate was 11.4 ($\pm$ S.E.M. 1.2) μ moles of propionate consumed/mg. of mitochondrial N/hr.

Statistical analysis of these results revealed that the effect of the mitochondrial supernatant was highly significant by the *t* test ($t = 4.404$ on 20 degrees of freedom; $P < 0.001$).

Fractionation of the mitochondrial supernatant. Examination of the mitochondrial supernatant revealed the presence of two stable factors that were capable of stimulating the rate of consumption of propionate when preincubated with washed mitochondria.

One of these was present in the non-diffusible

material after dialysis and could be recovered by fractionation with ammonium sulphate. The second factor was detected first in an ultrafiltrate of fresh mitochondrial supernatant, and subsequently in boiled mitochondrial supernatant.

The non-diffusible material was recovered in the following way. A 90 ml. portion of frozen (-20°)-and-thawed mitochondrial supernatant was dialysed overnight at 1° with stirring against three changes of 5 l. of 5 mM-potassium phosphate buffer, pH 7.4, containing GSH (0.1 mM). After centrifugation for 10 min. at 35000g (Spinco model L preparative ultracentrifuge) at 0°, the supernatant was recovered. Without further pH adjustment, the clear supernatant was treated with powdered ammonium sulphate to obtain three fractions. From the nomogram of Dixon (1953), the three precipitates recovered were 0–38% saturation, 38–68% saturation and 68–93% saturation. Each addition of ammonium sulphate occupied 20 min. and was accompanied by mechanical stirring, which was continued for a further 20 min. Precipitates were recovered by centrifugation for 10 min. at 35000g.

The sediments were dissolved in half the original volume of 5 mM-potassium phosphate buffer pH 7.4, containing GSH (0.1 mM), and dialysed overnight with stirring against three changes of 3 l. of the same buffer. After adding sucrose to give a concentration of 0.25 M, the contents of the dialysis sacs were tested for their ability to stimulate consumption of propionate by washed mitochondria, as described in the Materials and Methods section. Activity was found only in the fraction obtained between 38 and 68% saturation.

Table 1 shows the stimulation obtained with 38–68% and 40–60% saturated ammonium sulphate fractions of dialysed mitochondrial supernatants. In the two experiments shown, the lowest amounts correspond approx. to 0.5 ml. of the original mitochondrial supernatants from which they were derived. The use of higher amounts than those shown led to progressive inhibition in the rate of consumption of propionate.

In no case was a stimulation either by dialysed supernatant or by an ammonium sulphate fraction as great as that obtained with 0.5 ml. of fresh mitochondrial supernatant, and a diffusible factor was sought.

An ultrafiltrate of fresh mitochondrial supernatant was prepared by centrifugation in 0.25 in. (inflated diam.) cellophan dialysis tubing, as described by Samachson & Lederer (1958). Tubes were centrifuged for 45 min. at 600g in an International refrigerated centrifuge at 0–2° and the clear colourless ultrafiltrate was collected. The combined effects of ultrafiltrate and the 40–60% saturated ammonium sulphate fraction are shown in Table 2. In Table 3 is shown a further experiment in which the

Table 1. *Effect of ammonium sulphate fractions of dialysed mitochondrial supernatant on the rate of metabolism of propionate by aged sheep-liver mitochondria*

Mitochondria from 0.33 g. of liver were incubated for 36 min. at 37° with potassium propionate (5 mm) after aging for 19 min. at 37°. The effect of the mitochondrial supernatant is compared with that of $(\text{NH}_4)_2\text{SO}_4$ fractions isolated from dialysed mitochondrial supernatant. The lowest amount of protein in each case was derived from approximately the quantity of fresh mitochondrial supernatant employed (0.5 ml. of a 1:6 homogenate, corresponding to 0.08 g. of liver). Experimental conditions are described in the text and in the Materials and Methods section; the gas phase was $\text{O}_2 + \text{CO}_2$ (95:5).

Expt. no.	Mitochondrial protein added (mg.)	Supplementary protein added (mg.)	Mitochondria alone	Propionate consumed ($\mu\text{moles/mg. of mitochondrial N/hr.}$)		
				Mitochondria + fresh mitochondrial supernatant	Mitochondria + dialysed 38–68% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction	Mitochondria + dialysed 40–60% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction
3	2.07	Nil	4.1	—	—	—
	2.07	1.4	—	9.8	—	—
	2.07	1.4	—	—	6.6	—
	2.07	2.8	—	—	7.7	—
	2.07	5.6	—	—	8.2	—
5	1.55	Nil	7.7	—	—	—
	1.55	1.8	—	13.7	—	—
	1.55	1.9	—	—	—	7.8
	1.55	3.8	—	—	—	9.1
	1.55	7.7	—	—	—	10.3

Table 2. *Combined effects of an ammonium sulphate fraction of dialysed mitochondrial supernatant and an ultrafiltrate of fresh mitochondrial supernatant on the rate of metabolism of propionate by aged sheep-liver mitochondria (Expt. 6)*

Mitochondria (1.44 mg. of N) from 0.33 g. of liver were incubated for 36 min. at 37° in the presence of potassium propionate (5 mm) after incubation for 19 min. with the combinations of the $(\text{NH}_4)_2\text{SO}_4$ fraction and ultrafiltrate shown. Experimental details are given in the text and in the Materials and Methods section; the gas phase was $\text{O}_2 + \text{CO}_2$ (95:5).

40–60% Saturated $(\text{NH}_4)_2\text{SO}_4$ fraction added (mg. of protein/flask)	Vol. of ultrafiltrate/flask.....	Propionate consumed ($\mu\text{moles/mg. of mitochondrial N/hr.}$)		
		Nil	0.125 ml.	0.25 ml.
0.0		2.2	6.0	5.4
1.9		5.7	5.3	8.3
3.8		5.4	6.7	8.4*

* 0.5 ml. of the mitochondrial supernatant (2.6 mg. of N/ml.) from which the ultrafiltrate was derived led to a rate of 9.4 μmoles of propionate consumed/mg. of mitochondrial N/hr.

Table 3. *Combined effects of an ammonium sulphate fraction of dialysed mitochondrial supernatant and boiled fresh mitochondrial supernatant on the rate of metabolism of propionate by aged mitochondria from sheep liver (Expt. 9)*

Mitochondria (1.95 mg. of N) from 0.33 g. of liver were incubated for 36 min. at 37° in the presence of potassium propionate (5 mm) after incubation for 19 min. with combinations of the $(\text{NH}_4)_2\text{SO}_4$ fraction and boiled mitochondrial supernatant shown. Experimental details are given in the text and in the Materials and Methods section; the gas phase was $\text{O}_2 + \text{CO}_2$ (95:5).

40–60% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction added (mg. of protein/flask)	Vol. of boiled mitochondrial supernatant/flask...	Propionate consumed ($\mu\text{moles/mg. of mitochondrial N/hr.}$)		
		Nil	0.25 ml.	0.5 ml.
0.0		4.1	8.7	9.5
1.9		5.6	9.6	10.2
3.8		5.7	10.6	10.1*

* 0.5 ml. of the fresh mitochondrial supernatant (3.5 mg. of N/ml.) from which the boiled mitochondrial supernatant was derived led to a rate of 10.4 μmoles of propionate consumed/mg. of mitochondrial N/hr.

ultrafiltrate was replaced by boiled supernatant. The boiled supernatant was prepared from fresh mitochondrial supernatant by heating 10 ml. portions in test tubes for 5 min. in a boiling-water bath, chilling rapidly and centrifuging for 30 min.

at 105 000g in stainless-steel tubes in a Spinco ultracentrifuge at 0–2°. The clear yellow supernatant from this centrifugation was tested for activity. It is clear from Table 3 that a combination of boiled mitochondrial supernatant and the 40–60%

saturated ammonium sulphate fraction of dialysed mitochondrial supernatant was able to replace fresh mitochondrial supernatant in stimulating propionate consumption by aged mitochondria.

Fractionation of boiled mitochondrial supernatant. It is apparent from Tables 2 and 3 that the diffusible factor was quantitatively more significant in stimulating mitochondrial propionate metabolism, and this was fractionated further. The experiments leading to the identification of L-glutamate as the active factor were performed in the absence of the ammonium sulphate fraction.

It was found initially that passage of the boiled mitochondrial supernatant through a column of Dowex 1 (Cl⁻ form) quantitatively removed the stimulating factor, whereas passage through a column of Dowex 50 (Na⁺ form) did not. The preliminary fractionation was therefore performed by adsorption of the active factor on Dowex 1 (Cl⁻ form) followed by gradient elution with hydrochloric acid.

A 100ml. portion of the clear yellow supernatant prepared as described above from boiled mitochondrial supernatant was applied to a column (50cm. $\times$ 2cm.²) of Dowex 1 (Cl⁻ form; 8% cross-linked; 100–200 mesh) and washed through with 200ml. of water. The eluate and washings were discarded and the column was eluted at 1° with a gradient of dilute hydrochloric acid. The mixing chamber contained initially 1l. of glass-distilled water into which was introduced with stirring (magnetic) 0.1N-hydrochloric acid. Fractions (85 of 10ml. each) were collected with an automatic fraction cutter and assays performed in each fraction for total phosphate, ninhydrin-positive material (Moore & Stein, 1948), thiol groups and extinctions at 260m μ and 232m μ . On the basis of these assays the tubes were combined into ten groups, and after neutralization samples equivalent to 0.5 and 1.0ml. of the original boiled supernatant were tested for their ability to stimulate the metabolism of propionate by mitochondria. The results for the amount equivalent to 0.5ml. are shown in Table 4. Activity was found only in one of the groups (tubes 18–20), corresponding to the single ninhydrin-positive peak. The inhibition obtained in fractions 7 and 10 is not understood, but was confirmed for the amount equivalent to 1.0ml.

The material from a fresh preparation of fraction 4 was freeze-dried and applied in water as a streak to a sheet of Whatman no. 17 paper. The paper had previously been washed with 0.1% EDTA, with glass-distilled water until free from EDTA, and finally with ethanol–water (77:23, v/v), and then dried. The freeze-dried material was chromatographed (ascending) in 100% ethanol for 36hr. at 1°, the paper being clamped lightly between glass plates. During this time the solvent progressed

20 in. past the origin. After drying in air at 1° (7hr. in a stream of air) the paper was cut transversely into ten 2 in. strips and each strip eluted chromatographically with 50ml. of glass-distilled water. After neutralization of samples of the eluates these were tested for activity in amounts corresponding to 0.5ml. of fresh mitochondrial supernatant. The results (means of duplicate incubations) are shown in Table 4. Activity was confined to a single fraction, corresponding to the line of application of the material, and including virtually the whole of the ninhydrin-positive material (ninhydrin-sprayed longitudinal strip). This chromatographic step was included to separate the ninhydrin-positive material from any ethanol-soluble acids that may have been present.

Paper chromatography of a small portion of the paper eluate (fraction 1) with butan-1-ol–water–propionic acid (45.5:32:22.5, by vol.) solvent on Whatman no. 1 paper (Edwards, Gadsden, Carter & Edwards, 1959) revealed the presence of only glutamate and aspartate.

The glutamate and aspartate in the remainder of the paper eluate (fraction 1) were separated on the anion-exchange resin Amberlite IR4-B by the method of Consden, Gordon & Martin (1948). After concentration *in vacuo* to about 6ml. and adjustment with hydrochloric acid to pH 2.5, the sample was applied to the column (0.7cm. diam. $\times$ 40cm., previously equilibrated with 3mN-hydrochloric acid) and eluted at room temperature with 3mN-hydrochloric acid. Fractions (24, each of 10ml.) were collected with an automatic fraction cutter, and tubes containing glutamate or aspartate identified by spot test with ninhydrin (Cramer, 1954). On the basis of the spot tests, the eluate was combined in five fractions: pre-glutamate (tubes 1–2), glutamate (tubes 3–7), intermediate (tubes 8–11), aspartate (tubes 12–18) and post-aspartate (tubes 19–24). After neutralization, samples of the five fractions were tested for activity in stimulating propionate consumption at amounts equivalent to 1ml. of boiled mitochondrial supernatant. As shown in Table 4 activity was largely confined to the glutamate fraction.

The main bulk of the glutamate fraction (tubes 3–7) was then evaporated, first *in vacuo*, and then in a 15ml. conical centrifuge tube in a boiling-water bath under a stream of nitrogen, to a final volume of about 0.3ml. This solution was saturated with dry hydrogen chloride gas at 0°, stoppered and allowed to crystallize at –20° (Block, 1938). The crystalline material (about 10mg.) was washed with concentrated hydrochloric acid and dried *in vacuo* over solid sodium hydroxide. A portion of the crystalline material was compared with authentic L-glutamic acid hydrochloride (prepared in a similar way) for activity in stimulating the metabol-

Table 4. *Isolation of boiled mitochondrial supernatant factor stimulating the metabolism of propionate by aged sheep-liver mitochondria*

Mitochondria from 0.33 g. of liver (1.95 mg. of N in Expt. 9; 1.81 mg. of N in Expt. 11) were incubated for 36 min. at 37° in the presence of potassium propionate (5 mM) after incubation for 19 min. with the indicated fractions derived from boiled mitochondrial supernatant. The boiled mitochondrial supernatant employed to prepare the Dowex 1 (Cl⁻ form) eluate (Expt. 9) was derived from the mitochondrial supernatant from Expt. 8. Paper chromatography and Amberlite IR4-B eluate (Expt. 11) were prepared from the ninhydrin-positive fraction (fraction 4) from a similar Dowex 1 (Cl⁻ form) eluate prepared from the boiled mitochondrial supernatant of Expt. 10 and shown to be active. Experimental conditions were as described in the text and in the Materials and Methods section; the gas phase was O₂ + CO₂ (95:5).

Dowex 1 (Cl ⁻ form) eluate: gradient elution with HCl (Expt. 9)				Water eluate of paper chromatogram of Dowex 1 (Cl ⁻ form) fraction 4: Developed with 100% ethanol (Expt. 11)				Amberlite IR4-B eluate in 3mM-HCl of paper fraction 1 (Expt. 11)					
Combined fraction no.	Tube no.	Propionate consumed (μmoles/mg. of mitochondrial N/hr.)	Fraction no.	Distance from origin (in.)	Propionate consumed (μmoles/mg. of mitochondrial N/hr.)	Combined fraction no.	Tube no.	Propionate consumed (μmoles/mg. of mitochondrial N/hr.)	Identification	Combined fraction no.	Tube no.	Propionate consumed (μmoles/mg. of mitochondrial N/hr.)	Identification
No addition	—	4.1	No addition	—	9.8	No addition	—	9.8	—	No addition	—	9.8	—
1	1-6	4.3	1	0-2	15.0	1	1-2	9.6	Pre-glutamate	1	1-2	9.6	Pre-glutamate
2	7-13	4.0	2	2-4	10.4	2	3-7	14.8	Glutamate	2	3-7	14.8	Glutamate
3	14-17	5.0	3	4-6	9.0	3	8-11	11.0	Intermediate	3	8-11	11.0	Intermediate
4	18-20	10.2	4	6-8	9.0	4	12-18	11.6	Aspartate	4	12-18	11.6	Aspartate
5	21-29	4.4	5	8-10	8.8	5	19-24	10.9	Post-aspartate	5	19-24	10.9	Post-aspartate
6	30-32	2.4	6	10-12	8.7								
7	33-39	1.5	7	12-14	9.1								
8	40-53	2.7	8	14-16	9.0								
9	54-70	3.3	9	16-18	9.0								
10	71-85	0.3	10	18-20	8.8								
Fresh super- natant (0.5ml.)	—	10.4	Fresh super- natant (0.5ml.)	—	18.2	Fresh super- natant (0.5ml.)	—	18.2	—	Fresh super- natant (0.5ml.)	—	18.2	—

ism of propionate by washed sheep-liver mitochondria. The results are shown in Table 5.

The crystalline material was identified as L(+)-glutamic acid hydrochloride by paper chromatography (Edwards *et al.* 1959), paper electrophoresis (Frahn & Mills, 1964), melting point (169–171·5°, uncorrected, authentic material m.p. 170–172°, uncorrected), mixed melting point (168–171·5°, un-

corrected) and optical rotation. Calculated as glutamic acid $[\alpha]_D$ was +23° (c 0·315 in 1·0 N-hydrochloric acid; 0·5 dm. tube), whereas authentic L-glutamic acid gave $[\alpha]_D$ +32·6 ± 1° (c 1·0 in 1·0 N-hydrochloric acid; 0·5 dm. tube). The measurement of optical rotation was performed in the micro-cell (0·5 ml.) of a Hilger polarimeter (model M412) on a total weight of 1·98 mg. of the crystalline material. The net angle of rotation measured was only +0·036° (mean of 24 readings) and was probably subject to considerable error.

Effects of glutamate and protein on aging. To distinguish between the stimulating and protective effects of the two factors isolated an experiment was performed in which the effects were measured with and without preliminary incubation of the mitochondria in the absence of substrate. As in previous experiments the two factors were present with the mitochondria during the period of incubation without substrate. The results of the experiment (means of duplicate incubations) are given in Table 6.

It is clear that the protein fraction (38–68% saturated ammonium sulphate fraction) was virtually without effect on mitochondria that had not been aged, but exerted a protective effect against the aging process. Glutamate caused a marked stimulation of the rate at which propionate was metabolized either with or without aging, and the combination of both factors completely replaced the mitochondrial supernatant. The smaller effect of 2 μ moles of glutamate (0·33 mm) after 19 min. incubation without propionate was probably due to metabolism of a portion of the glutamate by the mitochondria during this period. (The concentrations of glutamate were chosen to illustrate this

Table 5. *Effect of L-glutamic acid hydrochloride and isolated crystalline hydrochloride on the metabolism of propionate by aged sheep-liver mitochondria (Expt. 11)*

Mitochondria (1·81 mg. of N) from 0·33 g. of liver were incubated for 36 min. at 37° in the presence of potassium propionate (5 mm) and either crystalline L-glutamic acid hydrochloride or the isolated crystalline material. The experimental conditions and the composition of the medium are described in the text and in the Materials and Methods section. In this as in other experiments, the materials being tested were incubated with mitochondria for 19 min. at 37° before propionate was added from the side arm. The gas phase was O₂ + CO₂ (95:5).

Addition	Propionate consumed (μ moles/mg. of mitochondrial N/hr.)
None	9·8
L-Glutamic acid hydrochloride (156 μ g.)	14·9
L-Glutamic acid hydrochloride (312 μ g.)	15·8
L-Glutamic acid hydrochloride (624 μ g.)	20·4
Isolated crystalline hydrochloride (312 μ g.)	15·7

Table 6. *Combined effects of an ammonium sulphate fraction of dialysed mitochondrial supernatant and L-glutamate on the rate of metabolism of propionate by sheep-liver mitochondria with and without aging*

Mitochondria (1·78 mg. of N) from 0·33 g. of sheep liver were incubated for 36 min. at 37° in the presence of potassium propionate (5 mm) after incubation for 2 or 19 min. in the presence of the combinations of L-glutamate and the (NH₄)₂SO₄ fraction shown. The 38–68% saturated (NH₄)₂SO₄ fraction was prepared from dialysed mitochondrial supernatant as described in the text, except that the final dialysis was performed against four changes of buffer instead of three. Experimental details are given in the text and in the Materials and Methods section; the gas phase was O₂ + CO₂ (95:5). Rates quoted are the means of duplicate incubations.

		Propionate consumed (μ moles/mg. of mitochondrial N/hr.)					
Additional protein present (mg.)	Additional glutamate present	Incubated for 2 min. in absence of propionate			Incubated for 19 min. in absence of propionate		
		Nil	2 μ moles	10 μ moles	Nil	2 μ moles	10 μ moles
0·0		8·4	13·1	13·2	2·4	8·6	12·6
10·7		8·6	13·4	13·5	8·0	11·3	13·6
21·3		9·0	13·4	13·5	7·4	11·5	13·7*

* Control flasks in which mitochondria were incubated with fresh mitochondrial supernatant (1 ml. containing 16·8 mg. of protein and 2·97 mg. of N) consumed propionate after aging for 2 and 19 min. respectively at rates of 13·1 and 12·8 μ moles/mg. of mitochondrial N/hr.

effect.) Though the combination of protein and glutamate completely protected the mitochondria against the aging process, this effect was very nearly achieved by glutamate alone. In an earlier experiment (not quoted) it was found that crystalline bovine albumin at comparable concentration could not replace the protein factor.

DISCUSSION

The lowered rate of metabolism of propionate by aged sheep-liver mitochondria as compared with aged nuclear-free homogenates may be seen by comparison of the results in Table 1 with rates given by Smith & Osborne-White (1965). The rates then obtained (2.4 μ moles/mg. of N/hr.) referred largely to nuclear-free homogenates containing about 3.5 mg. of N/ml. of homogenate. Neglecting incompleteness of homogenization this amounts to about 58 μ moles of propionate/g. of liver/hr. In the present experiments mitochondria from 0.33 g. of liver with about 1.7 mg. of N achieved a mean rate of 5.0 μ moles/mg. of N/hr. (Table 1) or 26 μ moles/g. of liver/hr. When supplemented with the mitochondrial supernatant representing 0.08 g. of liver (mean 1.55 mg. of N) this rate was increased to 11.4 μ moles/mg. of mitochondrial N/hr. or again about 58 μ moles/g. of liver/hr.

It has been shown (Smith & Osborne-White, 1965) that incubation of the nuclear-free homogenate for 19 min. in the absence of substrate caused a decrease of only about 6% in the rate at which propionate was metabolized, whereas similar treatment of isolated mitochondria led to the loss of about 60% (mean of three experiments 58%) of the capacity to metabolize propionate without aging. The present experiments demonstrate the existence of two factors in the soluble fraction responsible for stimulation of propionate metabolism and protection against aging. The non-diffusible factor appears to function solely as a protective agent against the aging process, whereas glutamate exerts a direct stimulatory effect on the rate of propionate metabolism by mitochondria. Glutamate also appears to be capable of protecting the mitochondria against

loss of activity by aging. The rate of consumption of propionate with glutamate alone (1.7 mm) was 92% of that in the presence of both factors. When both factors were present at suitable concentrations, there was no detectable loss of activity on aging.

The presence of free glutamate at relatively high concentration as compared with other amino acids has been demonstrated in the livers of a number of species, including sheep (Plaquet, Biserte & Boulanger, 1962). The interrelations of propionate and glutamate metabolism in aged sheep-liver mitochondria have been studied (Smith, Osborne-White & Russell, 1965).

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