A Continuous Recording Technique for the Measurement of Carbon Dioxide, and its Application to Mitochondrial Oxidation and Decarboxylation Reactions

BY D. G. NICHOLLS, D. SHEPHERD AND P. B. GARLAND

Department of Biochemistry, University of Bristol

(Received 25 October 1966)

1. A technique is described for continuously recording the concentration of carbon dioxide in mitochondrial suspensions. 2. The oxidation of palmitoylcarnitine by rat-liver mitochondria inhibits the oxidation of pyruvate and isocitrate, and stimulates the carboxylation of pyruvate. 3. These effects of palmitoylcarnitine oxidation are reversed by the uncoupling agent pentachlorophenol. 4. The effects of palmitoylcarnitine oxidation and pyruvate oxidation on the acylation of mitochondrial coenzyme A and reduction of nicotinamide nucleotides were measured. 5. Control mechanisms are discussed for the interactions between palmitoylcarnitine oxidation and the tricarboxylic acid cycle in rat-liver mitochondria.

Continuously recorded polarographic measurements of mitochondrial oxygen uptake have largely superseded manometric measurements since the introduction of the Clark type of oxygen electrode (Clark, Wolf, Granger & Taylor, 1953). A similarly convenient technique for the measurement of carbon dioxide has not been fully developed, and the carbon dioxide electrode systems described by Stow, Baer & Randall (1957) and Severinghaus & Bradley (1958) have a slow response time and a logarithmic relationship between the variable actually measured (pH) and the partial pressure of CO_2 . (CO_2 , H_2CO_3 , HCO_3^- and CO_3^{2-} are used throughout in their strict chemical sense, and the phrase 'total carbon dioxide' is used for the sum of the activities of all these species. Concentrations of bicarbonate solutions refer to total carbon dioxide content.) One purpose of the present paper is to describe a modified carbon dioxide electrode that has a response time sufficiently rapid to follow changes in the total carbon dioxide content of mitochondrial suspensions; another is to describe an analogue convertor presenting a recorder output that bears a linear relationship to the total carbon dioxide content of a mitochondrial suspension. By incorporating electrodes for oxygen and carbon dioxide in one incubation apparatus, and by using inhibitors for specific steps of the tricarboxylic acid cycle, it is possible to measure the respective rates of decarboxylating oxidations [e.g. oxidation of pyruvate, 2-oxoglutarate and $\text{L}_\alpha(+)$ -isocitrate] and non-decarboxylating oxidations (e.g. oxidation

of fatty acids and succinate) when both types of oxidation are occurring simultaneously.

The interactions between the tricarboxylic acid cycle and the oxidation of fatty acids, with particular reference to the identification of regulatory reactions and mechanisms, have mainly been investigated either at the level of the perfused rat liver (e.g. Struck, Ashmore & Wieland, 1965) or at the level of the purified enzyme (e.g. Utter & Keech, 1963). More recently the control of pyruvate oxidation (Bremer, 1966; Haslam, 1966; Stuart & Williams, 1967), acetoacetate and citrate synthesis (Shepherd, Yates & Garland, 1965) and pyruvate carboxylation (Lardy, Paetkau & Walter, 1965; Walter, Paetkau & Lardy, 1966) have been studied in mitochondrial preparations. Such studies can be facilitated by means of a dual-recording technique for oxygen and carbon dioxide and also extended to include the control of isocitrate and 2-oxoglutarate oxidation. Preliminary reports of part of this work have been published (Nicholls & Garland, 1966; Garland, Shepherd & Nicholls, 1966).

MATERIALS AND METHODS

Carbon dioxide electrode. This is similar in principle to those described by Stow *et al.* (1957) and Severinghaus & Bradley (1958). A flat-ended glass pH electrode (Radiometer type G.8520) was mounted in a Perspex cylinder machined to the shape shown in Fig. 1, and a reference electrode provided by a 3 in. length of 0.0012 in.-diam. silver wire electrolytically coated with AgCl by immersion

Fig. 1. Vertical section through the carbon dioxide electrode. Components are: *a*, electrode-retaining cap; *b*, Perspex electrode holder; *c*, electrolyte (0.1M-NaCl-0.1mM-NaHCO₃); *d*, Radiometer G.8520 pH electrode; *e*, rubber 'O'-ring; *f*, silicone rubber membrane; *g*, groove in the Perspex holder to accommodate *h*, the silver reference electrode. Further details are in the text.

Fig. 2. Vertical section through the incubation apparatus. Components are: *a*, carbon dioxide electrode; *b*, oxygen electrode; *c*, hole for additions; *d*, incubation medium; *e*, magnetic follower; *f*, water jacket.

in 0.1N-HCl and passage of 1mA for 30min. A groove at the tip held a rubber 'O'-ring that fastened a piece of silicone rubber membrane (type TC156; Esco Rubber Ltd., London, E. 8) over the pH-sensitive tip of the glass electrode. This type of silicone rubber has a slightly granular surface and there is no requirement for a layer of tissue paper or cellophan between the membrane and the glass electrode. The carbon dioxide electrode was mounted in a Perspex disk along with a Clark type of oxygen electrode similar to that described by Chappell (1961), and the combined electrode assembly was attached to a vertical rack-and-pinion device. The incubation chamber (Fig. 2) consisted of a length of precision-bore (30mm. internal diam.) glass tubing (Chance Bros., Smethwick), water-jacketed and closed at one end. The contents were stirred with a magnetic follower and magnetic stirrer (Rank Bros., Bottisham, Cambridge). The Perspex disk holding the electrodes was a close fit in the incubation chamber, and a 2mm.-diam. hole allowed additions. A convenient volume of incubation medium was 6ml. It is important that the tip of the Perspex electrode-containing cylinder should be a reasonably tight fit on the electrode itself, otherwise CO₂ can diffuse through the gap into the bulk of the electrolyte contained in the Perspex cylinder.

When assembling the carbon dioxide electrode the sequence of operations was: (i) the silicone rubber membrane over the rounded tip of the Perspex cylinder was attached, stretched and trimmed; (ii) 0.6ml. of an aqueous solution

of NaCl (0.1M) and NaHCO₃ (0.1mM) was added to the electrode compartment; (iii) 2μl. of a carbonic anhydrase solution (10mg./ml.) was mixed in; (iv) the glass electrode with the reference electrode fastened to it by two rubber bands was inserted, the reference electrode being positioned in its groove (Fig. 1); (v) the electrode-retaining caps were tightened to stretch the silicone rubber membrane over the end of the glass electrode.

Theory of the carbon dioxide electrode. CO₂ diffuses from the medium in the incubation chamber across the silicone rubber membrane and dissolves in, and equilibrates with, the thin layer of electrolyte between the membrane and the pH-sensitive tip of the glass electrode. The resulting hydration and dissociation reactions (1, 2 and 3) cause a change in pH of the electrolyte that is proportional to the change in log *p*CO₂ (Severinghaus & Bradley, 1958).

The second dissociation of carbonic acid (reaction 3) is negligible at the pH concerned (less than 7.5), and the hydration of carbon dioxide (reaction 1) is very much slower than the dissociation of carbonic acid (Gutfreund, 1965). Provided that the pH of the incubation remains effectively constant, the *p*CO₂ in the medium is proportional to the 'total carbon dioxide' concentration. The electrode sensitivity *S* is defined by:

$$S = \frac{-\Delta \text{pH}}{\Delta \log p\text{CO}_2} \quad (4)$$

where the pH referred to is that of the electrolyte layer in contact with the glass electrode. Severinghaus & Bradley (1958) showed that the value of *S* lies between 0.5 and 1.0,

Fig. 3. Circuit for an antilogarithmic amplifier. E_i and E_o are the input and output voltages respectively, d is a diode with logarithmic characteristics carrying a current I_f , R_L is the loop resistance, and A an operational amplifier with inputs labelled 1 and 2. It is assumed that there is negligible voltage difference between the inputs 1 and 2, and that the amplifier draws no current from them (Burr-Brown Research Corp. 1963).

Fig. 4. Forward characteristic of SX640 diode at 35° (ambient). Over the linear region, the change, D , of forward voltage required for a tenfold change in current was 93 mV.

its exact magnitude being determined by the concentration of bicarbonate in the electrode. It follows from eqn. (4) that:

$$\frac{p\text{CO}_2''}{p\text{CO}_2'} = \left(\frac{[\text{H}^+]''}{[\text{H}^+]'} \right)^{1/3} \quad (5)$$

where $p\text{CO}_2'$ and $p\text{CO}_2''$ are two arbitrary values of $p\text{CO}_2$, and $[\text{H}^+]'$ and $[\text{H}^+]''$ are the corresponding values of $[\text{H}^+]$ required to satisfy eqn. (4). The current available from the recorder output of a pH-meter is proportional to pH. It is therefore necessary to convert the output current into a function that is proportional to $[\text{H}^+]^{1/3}$ in order to obtain a linear relationship between $p\text{CO}_2$ and recorder response.

Analogue converter. The basic circuit for an antilogarithmic converter is shown in Fig. 3 (Cowell & Gordon, 1965). It consists essentially of an operational amplifier and a non-linear diode. The SX640 diode (Mullard Ltd., London, W.C. 1) possesses a forward characteristic that follows a logarithmic law over four decades of current in the range 10^{-9} – 10^{-5} A (Fig. 4). The change in forward voltage that is required to cause a tenfold change in forward current is constant in this range, and is assigned the value D . It is assumed (Burr-Brown Research Corp. 1963) that the loop gain of the operational amplifier is so high that the voltage between the amplifier inputs is negligible when negative feedback is applied. It follows that the voltage across the diode is equal to the input voltage E_i (Fig. 3). The amplifier draws virtually no current from its inputs, and therefore the entire diode current I_f must flow through the loop resistance R_L , resulting in an output voltage (E_o) of $-R_L \cdot I_f$. Within the operating range of the diode, a change in E_i is proportional to the corresponding change in $\log I_f$. Thus:

$$\Delta E_i = D \Delta \log E_o$$

Adopting two values of input voltage, E_i' and E_i'' respectively:

$$E_i'' - E_i' = D \log \left(\frac{E_o''}{E_o'} \right)$$

or

$$\frac{E_i'' - E_i'}{D} = \log \left(\frac{E_o''}{E_o'} \right) \quad (6)$$

The complete circuit for use with a Radiometer type 22 pH-meter is shown in Fig. 5. This pH-meter has a recorder output of -1.428 mA/pH unit, when operated on the 0–8 range, into an external load of 500Ω (RV1 of Fig. 5). The input voltage E_i to the antilogarithmic amplifier is the sum of E_p and E_b , where E_p is the voltage tapped off RV1 and E_b is a preset bias voltage. The diode has a resistance of not less than $400 \text{ k}\Omega$ under our operating conditions, and so RV1 was not shunted. RV1 is set so that ΔE_p is given by:

$$\Delta E_p = -\frac{D \Delta \text{pH}}{S} \quad (7)$$

As E_b is constant:

$$\Delta E_i = \Delta E_p = -\frac{D \Delta \text{pH}}{S}$$

or

$$\frac{\Delta E_i}{D} = -\frac{\Delta \text{pH}}{S}$$

Substituting in eqn. (6):

$$-\frac{\Delta \text{pH}}{S} = \log \left(\frac{E_o''}{E_o'} \right)$$

Taking antilogarithms of this equation:

$$\frac{E_o''}{E_o'} = \left(\frac{[\text{H}^+]''}{[\text{H}^+]'} \right)^{1/3} \quad (8)$$

Fig. 5. Final circuit for the antilogarithmic amplifier. Input terminals for connexion to pH-meter output are on the left, output to potentiometric recorder is on the right. A is a Fenlow A3 operational amplifier (Fenlow Electronics Ltd., Weybridge, Surrey). Component values: RV1, 500 Ω (Beckman type C precision multiturn potentiometer; Beckman Instruments Ltd., Glenrothes, Fife); RV2, 5k Ω ; RV3, 50k Ω ; R1, 100k Ω ; R2, 10k Ω ; R3, 5.6k Ω . The +1.5v supply (dry battery) with the potential divider formed by R2 and RV2 determines the bias potential E_b , and E_p is a fraction of the pH-meter output potential across RV1 and defined by eqn. (7). RV3 is a gain control, and M a point for monitoring the forward voltage across the diode SX640. E_i and E_o are the input and output voltages respectively.

Combining eqns. (5) and (8):

$$\frac{E_o''}{E_o'} = \frac{p\text{CO}_2''}{p\text{CO}_2'}$$

E_o' corresponds to a nominal value of $p\text{CO}_2'$ derived from the $p\text{CO}_2$ of the air-saturated incubation medium and the bias voltage E_b . In practice E_o' is set to about 270mv at the start of an experiment. As, during an experiment, E_o' and $p\text{CO}_2'$ are constants, it follows that E_o is proportional to $p\text{CO}_2$. If at the same time the pH of the incubation medium does not vary, then E_o is also proportional to the total carbon dioxide content.

The characteristics of the diode are temperature-sensitive, and the diode was mounted in a constant-temperature aluminium block (Mullard Ltd. 1963). The value of D for the particular diode was determined at the block temperature, and the electrode sensitivity S can be determined from a graph of ΔpH against $\Delta\log p\text{CO}_2$. D and S need not be determined, however, if RV1 is adjusted empirically until the best linearity is obtained between recorder response and total carbon dioxide concentration. A three-channel potentiometric recorder (Rikadenki; T.E.M. Sales Ltd., Crawley, Sussex) was used to record the oxygen-electrode current, the output from the antilogarithmic amplifier and the input voltage to the antilogarithmic amplifier at the monitor point shown in Fig. 5.

Operating procedure for the carbon dioxide electrode. Once the correct settings for RV1 and the bias voltage had been determined, the only daily manipulations involved replacing the electrolyte in the Perspex cylinder and occasionally

the silicone membrane, setting the gain control RV3 to obtain the desired overall sensitivity, checking the bias voltage and calibrating with standardized bicarbonate as described below.

Calibration of the carbon dioxide electrode. A known amount of CO_2 was generated in the incubation compartment in 6.0ml. of a solution of KCl (80mm), tris-chloride buffer, pH7.2 (20mm), MnCl_2 (2mm), NADP (1mm) and 1 unit of isocitrate dehydrogenase (EC 1.1.1.42) by adding $\text{L}_4(+)$ -isocitrate (0.0–5mm) that had been assayed spectrophotometrically in parallel experiments by the procedure of Siebert (1963). The oxidation of isocitrate proceeded rapidly to completion:

and further additions of isocitrate were made to complete the calibration. The electrode was then used to assay an air-equilibrated solution of NaHCO_3 to provide a secondary standard for further calibrations.

Reagents. Adenine nucleotides, NAD, NADP, CoASH, sodium pyruvate, 2-oxoglutaric acid, isocitrate dehydrogenase (EC 1.1.1.42), malate dehydrogenase (EC 1.1.1.37) and citrate synthase (EC 4.1.3.7) were purchased from Boehringer Corporation (London) Ltd., London, W. 5. Palmitoyl-DL-carnitine was synthesized from palmitoyl chloride (British Drug Houses Ltd., Poole, Dorset) and DL-carnitine (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) by the procedure of Bremer (1962), palmitoyl-CoA from palmitoyl chloride and CoA (Seubert, 1960), and

acetyl-CoA from acetic anhydride and CoA (Simon & Shemin, 1953). Oligomycin, rotenone and tris (Trizma base) were purchased from Sigma (London) Chemical Co. Ltd., London, S.W. 6. Pentachlorophenol and 2,4-dinitrophenol (British Drug Houses Ltd.) were twice recrystallized from water and dissolved in ethanol. Succinic acid, KH_2PO_4 , KCl, EDTA, MgCl_2 and sucrose were A.R. grade. Fluorocitrate (Morrison & Peters, 1954) was given by Sir Rudolph Peters, L_α (+)-isocitrate and lewisite oxide were given by Dr J. B. Chappell, atractyloside was given by Dr Sisto Luciano and carbonic anhydrase (EC 4.2.1.1; prepared by the method of Meldrum & Roughton, 1933) was given by Mr J. Heap. All aqueous solutions were prepared in water distilled twice from all-glass apparatus, and those solutions for use in mitochondrial experiments were adjusted to pH 7.2 with tris base or HCl. The preparation of mitochondria and fluorimetric assays for CoA and acetyl-CoA were as described by Garland, Shepherd & Yates (1965), except that in a number of cases acetyl-CoA was assayed by a fluorimetric modification (Shepherd & Garland, 1966) of the procedure of Ochoa, Stern & Schneider (1951). Changes in the reduction of mitochondrial nicotinamide nucleotides were continuously recorded with a laboratory-built double-beam spectrophotometer (Chance & Legallais, 1951) and a phase-sensitive detection system (Brookdeal Electronics Ltd., London, S.E. 13) based (Matthews, Faulkner & Green, 1965) on the detector described by Faulkner & Stannett (1964). Alternatively, the fluorimetric technique of Chance & Baltscheffsky (1958) was used.

Experimental design. Most experiments were performed with continuous recording of O_2 and CO_2 concentrations in 6-0 ml. of mitochondrial suspension. To measure the CoA and acetyl-CoA in mitochondrial 20 ml. mixtures were incubated without recording and additions were made and samples removed at time-intervals indicated by the previous smaller-scale experiment. After each addition the mitochondrial contents of CoA and acetyl-CoA changed rapidly to new steady-state values, which are presented in Table 2. Changes of the reduction of mitochondrial nicotinamide nucleotides were followed fluorimetrically or spectrophotometrically in a volume of 2.0 ml., and where the experiment was performed in parallel with an O_2 - and CO_2 -recording experiment the traces are shown on the same abscissa.

RESULTS

Performance of the carbon dioxide electrode

Response time. Recorder tracings after the addition of sodium hydrogen carbonate to the incubation medium are shown in Fig. 6. The time for 90% completion was approx. 23 sec. with carbonic anhydrase present in both the electrode and incubation compartments. Further additions of carbonic anhydrase to both compartments did not increase the speed of response, and diffusion across the membrane and into the electrolyte layer at the electrode tip was presumably rate-limiting. The 90%-response time on a logarithmic scale (i.e. directly from the pH-meter) was approx. 15 sec. Though by no means ideal, this response is adequate to follow events in mitochondrial suspensions,

Fig. 6. Recorder tracings demonstrating the response time of the electrode under various conditions. The incubation chamber contained 6 ml. of tris-chloride buffer, pH 7.2 (20 mM), KCl (80 mM), EDTA (1 mM) and NaHCO_3 ($50 \mu\text{M}$) at 37° , and at zero time $100 \mu\text{M}$ - NaHCO_3 was added (final concn.). In Expts. A and B, carbonic anhydrase ($20 \mu\text{g.}$) was present in the electrode compartment; it was omitted in Expts. C and D. In Expts. A and C, carbonic anhydrase ($50 \mu\text{g.}$) was also present in the incubation medium.

particularly if relatively low concentrations of mitochondria are used (approx. 1 mg. of protein/ml.). After an addition of bicarbonate the slope of the recorder tracing declined exponentially with time. By means of electronic differentiation of the rate of electrode response and addition to the already obtained response it would be theoretically possible to obtain a very much decreased 'computed' response time.

Neither intact nor ultrasonically disintegrated mitochondria could substitute for carbonic anhydrase in the incubation compartment, and this enzyme is presumably absent from mitochondria. According to Chappell & Crofts (1966), the mitochondrial membrane is impermeable to bicarbonate and the product of decarboxylation reactions that crosses the membrane is probably CO_2 , though Williams (1965) has strong evidence that this is not the case for rat-heart mitochondria. Owing to the differing activities of carbonic anhydrase in the electrode and incubation compartments it is conceivable that CO_2 could equilibrate more rapidly with the electrode compartment than with the incubation medium, thus causing an overshoot in the electrode response. This may explain the short-lived downward movement of the recorder tracing sometimes observed when a fast rate of oxidative decarboxylation was suddenly inhibited by the oxygen concentration's becoming zero.

Linearity. The relationship between completed recorder response and the total concentration of carbon dioxide (either generated from isocitrate or

Fig. 7. Relationship between completed recorder response and concentration of total carbon dioxide added. Conditions, apart from carbon dioxide concentration, were as for curve A of Fig. 6. Zero carbon dioxide concentration corresponds to air-saturated medium.

by addition of sodium hydrogen carbonate) of the incubation medium was linear over the range 0.03–0.5 mM. Non-linearity (Fig. 7) below 0.03 mM presented no problem, since the addition of mitochondria to the incubation medium invariably raised the total carbon dioxide concentration into the linear range. The relationship between rate of carbon dioxide production and slope of recorder tracing was linear (Fig. 8).

Sensitivity. Under the conditions obtaining for the data of Fig. 7 the noise level was less than 1 mV, corresponding to less than 1 μ M-total carbon dioxide.

Reproducibility. For constant sensitivity the electrode operating conditions must be the same at the start of each mitochondrial incubation (this follows from eqn. 9), and the simplest way to achieve this between each incubation was to withdraw briefly the electrode from contact with the silicone rubber membrane, thereby replacing the electrolyte layer at the electrode tip. In practice, changes of $\pm 5\%$ in sensitivity could occur between separate experiments under apparently the same operating conditions, and at the end of each experiment an addition of bicarbonate was made to calibrate the electrode.

Specificity. The electrode was insensitive to acetate (5 mM), butyrate (1 mM), octanoate (0.5 mM) and ammonium chloride (1 mM), all at pH 7.2. Potassium cyanide (1 mM) and sodium dithionite (1 mM) at pH 7.2 caused a large off-scale response. Changes in the pH of the incubation medium alter the sensitivity towards total carbon dioxide by altering the equilibria of reactions (3) and (2) directly and (1) indirectly. All the experiments described here were of short duration and any metabolically induced changes in the pH of the

Fig. 8. Relationship between the velocity of carbon dioxide production and the concentration of mitochondria. Carbon dioxide was generated by decarboxylation of L-(+)-isocitrate. The incubation medium (6.0 ml.) at 37° contained KCl (80 mM), tris-chloride buffer, pH 7.2 (20 mM), EDTA (1 mM), MgCl₂ (2 mM), AMP (2 mM), potassium phosphate buffer, pH 7.2 (5 mM), lewisite oxide (0.3 μ g./ml.), L-(+)-isocitrate (2.0 mM), L-malate (5 mM), carbonic anhydrase (50 μ g.) and 0.6–6.6 mg. of mitochondrial protein.

buffered incubation medium were without significant effect on the sensitivity of the system. Solutions of keto acids, and also ethanolic solutions, usually contained sufficient carbon dioxide to cause a small increase in the total carbon dioxide concentration when added to the incubation medium.

Oxidation of pyruvate

In mitochondria with coupled oxidative phosphorylation, pyruvate oxidation was abolished by the addition of palmitoyl-DL-carnitine (Figs. 9 and 10). In the experiment where L-malate was present (Fig. 10), there was carbon dioxide output before the addition of pyruvate. This rate was 50% inhibited by lewisite oxide (1 μ g./ml.) and abolished by rotenone (0.1 μ g./ml.), and was probably due in part to the oxidative decarboxylation of 2-oxoglutarate derived from endogenous glutamate by transamination with oxaloacetate formed from the L-malate. Unlike the carbon dioxide output associated with pyruvate oxidation, the malate-dependent rate was insensitive to palmitoyl-carnitine oxidation. During the phase of palmitoyl-carnitine oxidation shown in Figs. 9 and 10 there was an increased reduction of nicotinamide nucleotides and an increase in the mitochondrial acetyl-CoA/CoASH ratio (Table 1). Palmitoyl-carnitine oxidation also stimulated pyruvate carboxylation in mitochondria with coupled oxidative

Fig. 9. Pyruvate and palmitoyl-DL-carnitine oxidation by mitochondria: coupled phosphorylation with L-malate absent. The incubation medium (final volume 2.5 ml.) at 37° initially contained KCl (80 mM), tris-chloride buffer, pH 7.2 (20 mM), EDTA (1 mM), MgCl₂ (2 mM), AMP (2 mM), potassium phosphate buffer, pH 7.2 (10 mM), tris-malonate buffer, pH 7.2 (8 mM), and carbonic anhydrase (50 μg.). Additions were made of 3 mg. of mitochondrial protein at A, sodium pyruvate (5 mM) at B, palmitoyl-DL-carnitine (14 μM) at C. The broken trace labelled NAD(P)H₂ is the fluorescence at 420 mμ upwards with an excitation of 366 mμ, and represents reduced nicotinamide nucleotides (Chance & Baltscheffsky, 1958). The arrows labelled '2' and '4' correspond to the amounts observed in states 2 and 4 of Chance & Williams (1956), with palmitoylcarnitine as the substrate in state 4. The numbers in parentheses refer to oxygen uptake (mμg.atoms/min./mg.) and carbon dioxide output (mμmoles/min./mg.) at those points.

Fig. 10. Pyruvate and palmitoyl-DL-carnitine oxidation by mitochondria: coupled phosphorylation with L-malate (5 mM) present. Conditions, abbreviations and additions of mitochondria at A, pyruvate at B and palmitoyl-carnitine at C were otherwise as in Fig. 9.

phosphorylation (see below and Fig. 19). It is unlikely that the inhibition of pyruvate oxidation measured as abolition of carbon dioxide output was due to carbon dioxide fixation, since at 0.5 mM total carbon dioxide the rate of pyruvate carboxylation (Fig. 19) was only 40–50% of the activity of

Fig. 11. Pyruvate and palmitoyl-DL-carnitine oxidation by mitochondria: phosphorylation uncoupled with pentachlorophenol (5 μM), and L-malate absent. Conditions, abbreviations and additions of mitochondria at A, pyruvate at B and palmitoylcarnitine at C were otherwise as in Fig. 9.

Fig. 12. Pyruvate and palmitoyl-DL-carnitine oxidation by mitochondria: phosphorylation uncoupled with pentachlorophenol (5 μM), and L-malate (5 mM) present. Conditions, abbreviations and additions of mitochondria at A, pyruvate at B and palmitoylcarnitine at C were otherwise as in Fig. 9.

pyruvate oxidation (Fig. 9), and was probably even less at the lower carbon dioxide concentrations obtaining in the experiments of Figs. 9–12.

When oxidative phosphorylation was uncoupled with 5 μM-pentachlorophenol (Garbus & Weinbach, 1963), palmitoylcarnitine oxidation did not affect pyruvate oxidation in the presence of L-malate (Fig. 12), but caused a 50% inhibition when L-malate was absent (Fig. 11). In each case mitochondrial nicotinamide nucleotides were less reduced than in the coupled state. The lower and upper reference extents of nicotinamide nucleotide reduction correspond respectively to the substrate-depleted uncoupled state, when NADH₂ and

Table 1. *CoASH and acetyl-CoA in mitochondria during the oxidation of pyruvate and palmitoyl-DL-carnitine*

Each pair of lines represents one experiment where a 20 ml. mixture was incubated under conditions otherwise similar to those of the corresponding Figure, and three samples were removed for assay after the addition of pyruvate and a further three were removed after the addition of palmitoylcarnitine. Mean values are given. PmCn, Palmitoylcarnitine; PCP, pentachlorophenol.

Additions	Corresponding Figure no.	CoASH (mμmole/mg. of protein)	Acetyl-CoA (mμmole/mg. of protein)	Acetyl-CoA CoASH ratio
Pyruvate	9	0.38	0.68	1.79
Pyruvate+ PmCn	9	0.15	0.70	4.66
Malate+ pyruvate	10	0.39	0.08	0.20
Malate+ pyruvate+ PmCn	10	0.14	0.24	1.72
PCP+ pyruvate	11	0.33	0.55	1.73
PCP+ pyruvate+ PmCn	11	0.15	0.55	3.65
PCP+ malate+ pyruvate	12	0.78	<0.05	0.06
PCP+ malate+ pyruvate+ PmCn	12	0.35	0.10	0.29

Fig. 13. Pyruvate and palmitoyl-CoA oxidation by mitochondria. Conditions were as in Fig. 9 initially except that L-malate (5mM) was present and the volume was 6.0ml. Additions were made of 6mg. of mitochondrial protein at A, sodium pyruvate (5mM) at B, palmitoyl-CoA (20μM) at C and DL-carnitine (2.5mM) at D.

Fig. 14. Pyruvate and *n*-octanoate oxidation by mitochondria. Conditions were as in Fig. 9 initially except that L-malate (5mM) was present and the volume was 6.0ml. Additions were made of 6mg. of mitochondrial protein at A, sodium pyruvate (5mM) at B and potassium *n*-octanoate (0.2mM) at C.

NADPH₂ are undetectable in mitochondrial extracts, and to the state when palmitoylcarnitine is oxidized in state 4 (Chance & Williams, 1956) and about 70% of the NAD and over 95% of the NADP in mitochondrial extracts are reduced (D. Shepherd & P. B. Garland, unpublished work). Values for mitochondrial acetyl-CoA and CoASH corresponding to the experiments of Figs. 11 and 12 are shown in Table 1.

The carnitine-dependent oxidation of palmitoyl-CoA (Fritz & Yue, 1963) inhibited pyruvate oxidation (Fig. 13), as did the carnitine-independent oxidation of octanoate (Fig. 14). The oxidation of butyrate caused a 50% inhibition. In each case the

inhibitory effects of the oxidation of these fatty acids in the presence of L-malate (5mM) were abolished by pentachlorophenol (5μM) and not restored by oligomycin (5μg./ml.) and ATP (4mM). Walter *et al.* (1966) have reported that octanoate inhibits pyruvate oxidation by rat-liver mitochondria.

Oxidation of L₂(+)-isocitrate

In the presence of lewisite oxide, the oxidation of isocitrate proceeds to 2-oxoglutarate, with a value of unity for the ratio $-\Delta O/\Delta CO_2$, and the

Fig. 15. Oxidation of isocitrate by mitochondria. Conditions were as in Fig. 9 initially, except that lewisite oxide ($0.3 \mu\text{g./ml.}$) was present and the volume was 6.0 ml. Additions were made of 6 mg. of mitochondrial protein at A, $L_4(+)$ -isocitrate (2.0 mM) at B and L-malate (5 mM) at C.

Fig. 17. Oxidation of isocitrate and palmitoyl-DL-carnitine by mitochondria with uncoupled phosphorylation. Pentachlorophenol ($5 \mu\text{M}$) was present initially; otherwise conditions, abbreviations and additions of mitochondria at A, isocitrate at B and palmitoylcarnitine at C were as in Fig. 16.

Fig. 16. Oxidation of isocitrate and palmitoyl-DL-carnitine by mitochondria with coupled phosphorylation. Conditions were as in Fig. 9 initially, except that lewisite oxide ($0.3 \mu\text{g./ml.}$) and L-malate (5 mM) were present and the volume was 6.0 ml. Additions were made of 6 mg. of mitochondrial protein at A, $L_4(+)$ -isocitrate (2.0 mM) at B and palmitoyl-carnitine ($20 \mu\text{M}$) at C. The broken trace is from a double-beam spectrophotometric recording at $340\text{--}380 \text{ m}\mu$, and an upward deflexion represents nicotinamide nucleotide reduction $[\text{NAD(P)H}_2]$. The amounts corresponding to states 2 and 4 are indicated (Chance & Williams, 1956).

interpretation of rates of oxidation is not complicated by further operation of the tricarboxylic acid cycle. Isocitrate oxidation in substrate-depleted mitochondria requires L-malate (Chappell, 1964a), and this requirement, which has been attributed to the activation of a permease for isocitrate (Chappell, 1964b), is demonstrated in Fig. 15. The oxidation of isocitrate was $80\text{--}100\%$ inhibited by the oxidation of palmitoylcarnitine in mitochondria with

coupled oxidative phosphorylation (Fig. 16) and this effect was largely removed (Fig. 17) by pentachlorophenol ($5 \mu\text{M}$). The rate of isocitrate oxidation alone was almost twice as fast when oxidative phosphorylation was uncoupled (Figs. 16 and 17). Changes in the reduction of mitochondrial nicotinamide nucleotides were followed spectrophotometrically in separate experiments parallel to those of Figs. 16 and 17, and are shown superimposed in Figs. 16 and 17. The extensive reduction of nicotinamide nucleotides caused by palmitoylcarnitine oxidation was considerably decreased when oxidative phosphorylation was uncoupled with pentachlorophenol ($5 \mu\text{M}$).

Oxidation of 2-oxoglutarate

In the absence of phosphate and the presence of an uncoupling agent, the oxidation of oxoglutarate (reaction 10) is inhibited (Hunter, 1951; Borst & Slater, 1960; Chappell, 1961), and under such conditions (Table 2, Expt. 1) the activity of succinyl-CoA synthetase (reaction 11) is thought to be rate-limiting:

Table 2. *Effect of palmitoylcarnitine on 2-oxoglutarate oxidation*

Each experiment was performed at 37° with 6 ml. of air-saturated medium containing initially: KCl (80mm), MgCl₂ (4mm), EDTA (1mm), tris-chloride buffer, pH7.2 (20mm), tris-malonate buffer, pH7.2 (8mm), mitochondrial protein (6mg.), carbonic anhydrase (20μg.) and either AMP (2mm) or pentachlorophenol (5μm). Further additions were made as described in the Table: 2-oxoglutarate (4mm), pyruvate (4mm), P_i (5mm), palmitoyl-DL-carnitine (30μm). The rates listed are corrected for the endogenous rate observed in the absence of added substrate. PmCn, Palmitoylcarnitine; PCP, pentachlorophenol.

Expt. no.	Additions	Rate of CO ₂ evolution (mμmoles/min./ mg. of protein)	Rate of O ₂ uptake (mμg.atoms/min./ mg. of protein)
1	2-Oxoglutarate + PCP	4	10
	2-Oxoglutarate + PCP + P _i	35	33
2	2-Oxoglutarate + P _i	33	45
	2-Oxoglutarate + P _i + PmCn	21	85
	2-Oxoglutarate + P _i + PCP + PmCn	20	85
3	2-Oxoglutarate + PCP	4	10
	2-Oxoglutarate + PCP + PmCn	4	36
	2-Oxoglutarate + PCP + PmCn + P _i	30	68
	2-Oxoglutarate + PCP + P _i	35	33
4	2-Oxoglutarate + PCP	3	5
	2-Oxoglutarate + PCP + pyruvate	6	9
	2-Oxoglutarate + PCP + pyruvate + P _i	35	38
5	Pyruvate + PCP	10	15
	Pyruvate + PCP + 2-oxoglutarate	10	14
	Pyruvate + PCP + 2-oxoglutarate + P _i	37	43

Fig. 18. Control by ADP of the oxidative decarboxylation of 2-oxoglutarate. The incubation medium (final volume 6ml.) at 37° initially contained KCl (80mm), tris-chloride buffer, pH7.2 (20mm), EDTA (1mm), MgCl₂ (2mm), potassium phosphate buffer, pH7.2 (10mm), tris-malonate buffer, pH7.2 (5mm), and carbonic anhydrase (8μg./ml.). Additions were made of 6mg. of mitochondrial protein at A, 2-oxoglutarate (4mm) at B and ADP (0.146mm) at C. Respiratory control ratios, defined as the respiration rate during ADP stimulation divided by the rate after stimulation (Chance & Williams, 1956), were 4.8 for oxygen uptake and 5.3 for carbon dioxide output. The ADP/O ratio was 3.74 and the ADP/CO₂ ratio 3.34. The ratio of the rate of oxygen uptake to the rate of carbon dioxide release was 0.97.

presence of phosphate and an uncoupling agent, the oxidation of pyruvate and oxoglutarate were more nearly additive (Table 2, Expts. 4 and 5) and it seems likely that pyruvate dehydrogenase, succinyl-CoA synthetase and 2-oxoglutarate dehydrogenase all have access to the same pool of intramitochondrial CoA.

When oxoglutarate oxidation was restricted by the absence of phosphate in the presence of an uncoupling agent, palmitoylcarnitine oxidation was partially inhibited (Table 2, Expt. 3). Both in the absence and presence of an uncoupling agent, palmitoylcarnitine oxidation caused a 30% inhibition of oxoglutarate oxidation (Table 2, Expt. 2).

The experiment shown in Fig. 18 is included to demonstrate the validity of the recording electrode system for CO₂. The values for respiratory control or phosphorylation efficiency were very similar when calculated from either the oxygen uptake or carbon dioxide output during a phase of ADP phosphorylation.

Carboxylation of pyruvate

In mitochondria this reaction is catalysed by pyruvate carboxylase (EC 6.4.1.1) with acetyl-CoA as an obligatory activator (Keech & Utter, 1963; Henning & Seubert, 1964):

Under these particular conditions (Table 2, Expt. 4) the further addition of pyruvate did not cause a further rise in carbon dioxide output. In the

This enzyme is considered to be essential for the reversal of glycolysis from pyruvate, and it has been suggested that its activity is regulated in the intact cell by changes in the concentration of acetyl-CoA (Utter, Keech & Scrutton, 1964; Krebs, 1964; Wieland & Weiss, 1963; Tubbs & Garland, 1964). An increase in the acetyl-CoA content of rat-liver mitochondria occurs during the oxidation of palmitate, palmitoyl-CoA or palmitoylcarnitine (Garland *et al.* 1965; Shepherd *et al.* 1965), and it could be expected that pyruvate carboxylase would be activated under such conditions. In the experiment of Fig. 19 mitochondria were incubated with 0.5 mM-bicarbonate, and lewisite oxide and fluorocitrate were added to restrict decarboxylation reactions. Under these conditions the oxidation of palmitoylcarnitine caused a disappearance of carbon dioxide. This effect was dependent on the presence of pyruvate and abolished if pentachlorophenol (5 μ M) was also present. Succinate (5 mM) could not substitute for palmitoylcarnitine and it is concluded that the stimulation of carbon dioxide uptake during palmitoylcarnitine oxidation was due to activation of pyruvate carboxylase by acetyl-CoA. The observed rate of carbon dioxide fixation was approx. 10 m μ moles/min./mg. of mitochondrial protein and, on the basis of 60 mg. of mitochondrial protein/g. of fresh liver (Scholz &

Bücher, 1965), would correspond to 600 m μ moles/g. of fresh liver/min. The concentration of bicarbonate in our experiments was 0.5 mM, whereas the K_m for bicarbonate is 1–2 mM at pH 7.7 for the rat-liver enzyme (Henning & Seubert, 1964), and is 0.98 mM for the chicken-liver enzyme at pH 7.4 (Utter & Keech, 1963).

Walter *et al.* (1966) reported that oxidation of octanoate by rat-liver mitochondria caused a small (20%) stimulation of pyruvate carboxylation, compared with the rate when pyruvate alone was the substrate. L-Malate was not added to the incubation medium in their experiments, and it is possible that the intramitochondrial accumulation of acetyl-CoA that can occur when oxaloacetate cannot be generated from malate during pyruvate oxidation (Table 1) had stimulated pyruvate carboxylase sufficiently to mask any large effect of octanoate oxidation. Despite this, oxidation of octanoate caused an eightfold stimulation of acetoacetate synthesis (Table IV in Walter *et al.* 1966), and it can be concluded from their data that pyruvate carboxylase can be stimulated by lower intramitochondrial concentrations of acetyl-CoA than those required for acetoacetate synthesis.

DISCUSSION

Carbon dioxide electrode. The response time and sensitivity of the electrode system have been adequate for studying carbon dioxide output and uptake by mitochondria, and for many purposes this procedure may be preferable to manometry or to the measurement of $^{14}\text{CO}_2$ release from ^{14}C -labelled substrates, unless these are also continuously recorded (e.g. Williams, 1965).

Interactions between oxidation of palmitoylcarnitine and the tricarboxylic acid cycle. Observations made on the kinetic behaviour of biological systems more complex than isolated soluble enzymes rarely permit the underlying mechanism to be stated with certainty. At best, the behaviour of the more complex system can be considered consistent with the known kinetic properties of the enzymes involved. In our present experiments on pyruvate carboxylation and isocitrate oxidation, the effects of palmitoylcarnitine oxidation (Table 3) are consistent with the kinetic properties of pyruvate carboxylase from liver (Keech & Utter, 1963; Henning & Seubert, 1964) and the NAD-dependent isocitrate dehydrogenase from various sources (Chen & Plaut, 1962, 1963; Hathaway & Atkinson, 1963; Zanwal, Zink & Stachow, 1964; Goebell & Klingenberg, 1964). However, the situation with isocitrate oxidation is complicated by the presence of both an NAD- and also an NADP-dependent isocitrate dehydrogenase in rat-liver mitochondria. Goebell & Klingenberg (1964) reported specific

Fig. 19. Pyruvate carboxylation and palmitoyl-DL-carnitine oxidation by mitochondria. The incubation medium (final volume 6.0 ml.) at 37° contained KCl (80 mM), tris-chloride buffer, pH 7.2 (20 mM), EDTA (1 mM), MgCl_2 (2 mM), AMP (2 mM), potassium phosphate buffer, pH 7.2 (10 mM), sodium pyruvate (5 mM), KHCO_3 (0.5 mM), lewisite oxide (0.3 $\mu\text{g./ml.}$) and fluorocitrate (10 μM). Additions were made of 18 mg. of mitochondrial protein at A and palmitoylcarnitine (0.1 mM) at B.

Table 3. *Palmitoylcarnitine oxidation in intact rat liver mitochondria: comparison between its action on regulatory enzymes and the currently proposed mechanisms*

NAD(P) represents NAD and NADP as measured spectrophotometrically or fluorimetrically, and deductions with regard to NAD or NADP cannot be drawn unless it is assumed that they change qualitatively in the same direction. References: ¹Bremer (1966); ²Haslam (1966); ³Garland (1964); ⁴Garland & Randle (1964); ⁵this paper (Figs. 10 and 12); ⁶this paper (Table 1); ⁷Hathaway & Atkinson (1965); ⁸Shepherd & Garland (1966); ⁹Shepherd *et al.* (1965); ¹⁰Sauer & Erfle (1966); ¹¹Klingenberg & Slenczka (1959); ¹²Danielson & Ernster (1963); ¹³Chen & Plaut (1963); ¹⁴Goebell & Klingenberg (1964); ¹⁵Hathaway & Atkinson (1963); ¹⁶Zanwal *et al.* (1964); ¹⁷this paper (Figs. 16 and 17); ¹⁸D. Shepherd & P. B. Garland (unpublished work); ¹⁹Keech & Utter (1963); ²⁰Henning & Seubert (1964); ²¹this paper (Fig. 19).

	Proposed mechanism of regulation	Observed effect of palmitoyl-carnitine oxidation on enzyme activity in intact mitochondria	Observed effect of palmitoylcarnitine oxidation on proposed regulatory metabolites
Pyruvate dehydrogenase (malate present)	Inhibition by increased reduction of NAD ^{1, 2, 3} and by increased acetyl-CoA/CoASH ratio ^{1, 2, 4}	Coupled: inhibition ^{1, 2, 5} Uncoupled: no inhibition ⁵	Extensive reduction of NAD(P) ⁵ ; increased acetyl-CoA/CoASH ratio ⁶ Slight reduction of NAD(P) ⁵ ; low acetyl-CoA/CoASH ratio ⁶
Citrate synthase	Inhibition by increased phosphorylation of adenine nucleotides ^{7, 8}	Coupled: inhibition ⁹ Uncoupled: no inhibition ⁹	Phosphorylation occurs Phosphorylation absent
Acetoacetate-synthesis system (malate present)	Activation by increased acetyl-CoA/CoASH ratio ^{9, 10}	Coupled: activation ⁹ Uncoupled: inhibition ⁹	Increased acetyl-CoA/CoASH ratio ⁹ Low acetyl-CoA/CoASH ratio ⁹
Isocitrate dehydrogenase (NAD- and NADP-linked)	Inhibition by increased reduction of NADP (via an energy-dependent transhydrogenase) ^{11, 12} or of NAD ¹³ , and by increased phosphorylation of adenine nucleotides ^{13, 14, 15, 16}	Coupled: inhibition ¹⁷ Uncoupled: slight inhibition ¹⁷	Extensive reduction of NAD(P) ¹⁷ ; high NADPH ₂ /NADP ratio ¹⁸ ; phosphorylation occurs Slight reduction of NAD(P) ¹⁷ ; low NADPH ₂ /NADP ratio ¹⁸ ; phosphorylation absent
Pyruvate carboxylase	Activation by increased acetyl-CoA concn. and by ATP concn. ^{19, 20}	Coupled: activation ²¹ Uncoupled: no activation	High concn. of acetyl-CoA ^{6, 9} ; phosphorylation occurs Low concn. of acetyl-CoA ^{6, 9} ; phosphorylation absent

activities of 15 μ moles/min./mg. of mitochondrial protein for the NAD-dependent enzyme, and 33 μ moles/min./mg. for the NADP-dependent enzyme from rat liver. Neither alone would account for the observed rates of isocitrate oxidation by mitochondria and the relative contribution of each has not been assessed. The complex kinetic properties of the NAD-dependent enzyme suggest a regulatory role, but inhibition of isocitrate oxidation requires that both enzymes be inhibited. The high degree of reduction of mitochondrial NADP during palmitoylcarnitine oxidation (D. Shepherd & P. B. Garland, unpublished work) may serve to regulate the NADP-dependent isocitrate dehydrogenase.

Conclusions on the mechanism of control of pyruvate dehydrogenation cannot be drawn in the

absence of data on the kinetic behaviour of pyruvate dehydrogenase from rat-liver mitochondria, and our attempts to obtain such information have been frustrated by the low yields (less than 5%) of activity remaining after disruption of mitochondria. By analogy with the properties of pyruvate dehydrogenase from pig-heart mitochondria (Garland & Randle, 1964; Garland, 1964), it could be expected that the activity of the enzyme would be regulated by the concentration of its inhibitory products, acetyl-CoA and NADH₂. During short-term mitochondrial incubations, the total mitochondrial content of nicotinamide nucleotides (Klingenberg & Slenczka, 1959) and of CoASH (Garland *et al.* 1965) remains constant, and accumulation of NADH₂ or acetyl-CoA requires a fall in the concentration of NAD and CoASH. For this

reason measurements of NAD, NADH₂, CoASH and acetyl-CoA in mitochondria cannot easily distinguish between the possibilities that pyruvate dehydrogenase is controlled by product inhibition or, as suggested by Bremer (1966) and Haslam (1966), by the lack of NAD or CoASH. If, as with pig-heart pyruvate dehydrogenase, product inhibition were competitive with respect to the appropriate substrate (i.e. acetyl-CoA versus CoASH, NADH₂ versus NAD), both types of control mechanism could coexist and complement each other. However, there are valid reasons for discarding the idea that lack of NAD or CoASH alone can account for the inhibition of pyruvate oxidation caused by palmitoylcarnitine oxidation. First, the complete inhibition of pyruvate oxidation is not

matched by a similar inhibition of oxoglutarate oxidation despite their common requirements for NAD and CoASH. Secondly, the fall in CoASH content (Table 1) during inhibited pyruvate oxidation is small (50–60%) compared with the degree of inhibition (100%). Thirdly, although at least 40% of the total mitochondrial NAD is available to the oxidation of pyruvate and malate, only 20% is reduced during palmitoylcarnitine oxidation in state 3 (Garland, Shepherd, Nicholls, Yates & Light, 1967). It follows that unless a number of assumptions are made about 'compartmentation' the experimental data do not support the idea that palmitoylcarnitine oxidation inhibits pyruvate oxidation solely by removal of NAD and CoASH.

The regulatory effects of palmitoylcarnitine

Scheme 1. Metabolic control points in rat-liver mitochondria oxidizing palmitoylcarnitine. In mitochondrial preparations with coupled oxidative phosphorylation (i.e. energy conservation coupled with electron transfer), palmitoylcarnitine oxidation under the appropriate experimental conditions (see the text) effected control at the following points: (a) pyruvate carboxylation (stimulated), (b) pyruvate oxidation (inhibited), (c) acetoacetate synthesis (stimulated), (d) citrate synthesis (inhibited), (e) isocitrate oxidation (inhibited). The malate-dehydrogenase reaction (f) is shown as reversible, though during pyruvate carboxylation to oxaloacetate there is a net conversion of oxaloacetate into malate (Lardy *et al.* 1965). In mitochondrial preparations with uncoupled oxidative phosphorylation, the effects of palmitoylcarnitine oxidation at points (a), (b), (c), (d) and (e) were abolished, and the malate-dehydrogenase reaction proceeded in the direction of oxaloacetate formation. That part of the tricarboxylic acid cycle between 2-oxoglutarate and malate is represented in the scheme by the broken line.

oxidation on pyruvate oxidation, pyruvate carboxylation, isocitrate oxidation, and also citrate and acetoacetate synthesis (Shepherd *et al.* 1965), are dependent on the integrity of the mechanisms that couple phosphorylation to electron transport, since in each case the effects of palmitoylcarnitine oxidation largely or completely disappear in the presence of pentachlorophenol. The proposed regulatory mechanisms in each case and the effects of pentachlorophenol are listed in Table 3. In each case it has been assumed that the regulatory mechanism is operative on an intramitochondrial enzyme and that the penetration of added substrates (pyruvate, malate, isocitrate, 2-oxoglutarate) into mitochondria is not rate-limiting or, under the conditions of these experiments, a point of metabolic regulation. With pyruvate, this assumption finds support in the demonstration that pyruvate can still penetrate to the site of pyruvate carboxylase during palmitoylcarnitine oxidation. However, it is not currently known whether the rate of isocitrate penetration in the presence of 5 mM-L-malate is in excess of the rate of isocitrate oxidation.

In all our experiments the highest rates of oxygen uptake exhibited by mitochondria with coupled oxidative phosphorylation were one-half to two-thirds of the rates obtainable with 5 mM-succinate as the substrate. It would thus appear that the non-additive behaviour of palmitoylcarnitine oxidation with pyruvate or isocitrate oxidation is not due to saturation of a reaction lying between the site of action of pentachlorophenol and the phosphorylation of ADP.

The physiological significance of the effects of palmitoylcarnitine oxidation in isolated rat-liver mitochondria can be assessed with regard to the possibility that similar effects occur in the liver *in vivo* during high rates of fatty acid oxidation (e.g. starvation, alloxan-diabetes). Stimulation of gluconeogenesis, inhibition of pyruvate oxidation, inhibition of citrate synthesis and stimulation of acetoacetate synthesis during high rates of fatty acid oxidation in the intact rat, and also in isolated perfused liver, are well documented (see reviews by Greville, 1966; Williamson, 1967), and the interactions (Scheme 1) now put forward for isolated rat-liver mitochondria on the basis of the results reported here and by Bremer (1966), Haslam (1966) and Shepherd *et al.* (1965) would appear to be valid for the situation *in vivo*. Despite the apparent inhibition of citrate synthesis when high rates of fatty acid oxidation obtain *in vivo*, the citrate content of liver does not fall, and in fact may increase (Parmeggiani & Bowman, 1963; Tubbs & Garland, 1964; Kalkhoff, Hornbrook, Burch & Kipnis, 1966). The observation that palmitoylcarnitine oxidation inhibits isocitrate oxidation may explain the maintenance of normal

or elevated citrate concentrations in the intact liver.

The effects of palmitoylcarnitine oxidation on the tricarboxylic acid cycle are energy-dependent in that they are abolished by uncoupling concentrations of pentachlorophenol. There is no particular reason to suppose that the energy-dependent mechanisms that control pyruvate oxidation and carboxylation, citrate and acetoacetate synthesis and isocitrate oxidation should all be set to respond to a similar extent in all situations, and the two states used in our experiments (palmitoylcarnitine oxidation at maximal rates in mitochondria with coupled or uncoupled oxidative phosphorylation) represent oversimplified extremes. A physiological equivalent to an uncoupling agent is a biosynthetic process, and the situation in the intact liver would be modified by the rates of energy utilization (e.g. for gluconeogenesis and urea synthesis) and the rates at which energy could be derived from the oxidation of fatty acids and the carbon catabolism of amino acids.

We are indebted to the Medical Research Council for research expenses and support for D.G.N., to the Royal Society for a Government grant to aid the construction of the double-beam spectrophotometer, and to Miss J. Matthews for skilled technical assistance.

REFERENCES

- Borst, P. & Slater, E. C. (1960). *Biochim. biophys. Acta*, **41**, 170.
- Bremer, J. (1962). *J. biol. Chem.* **237**, 3628.
- Bremer, J. (1966). *Biochim. biophys. Acta*, **116**, 1.
- Burr-Brown Research Corp. (1963). *Handbook of Operational Amplifiers*. Tucson, Ariz.: Burr-Brown Research Corp.
- Chance, B. & Baltscheffsky, H. (1958). *J. biol. Chem.* **233**, 736.
- Chance, B. & Legallais, V. (1951). *Rev. sci. Instrum.* **22**, 627.
- Chance, B. & Williams, G. R. (1956). *Advanc. Enzymol.* **17**, 65.
- Chappell, J. B. (1961). In *Biological Structure and Function*, vol. 2, p. 71. Ed. by Goodwin, T. W. & Lindberg, O. London: Academic Press (Inc.) Ltd.
- Chappell, J. B. (1964a). *Biochem. J.* **90**, 225.
- Chappell, J. B. (1964b). *Abstr. 6th int. Congr. Biochem.*, New York, vol. 8, p. 625.
- Chappell, J. B. & Crofts, A. R. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 293. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Chen, R. F. & Plaut, G. W. E. (1962). *Fed. Proc.* **21**, 244.
- Chen, R. F. & Plaut, G. W. E. (1963). *Biochemistry*, **2**, 1023.
- Clark, L. C., jun., Wolf, R., Granger, F. & Taylor, Z. (1953). *J. appl. Physiol.* **6**, 189.
- Cowell, T. K. & Gordon, M. (1965). *Electron. Engng.* **37**, 180.
- Danielson, L. & Ernster, L. (1963). *Biochem. biophys. Res. Commun.* **10**, 91.

- Faulkner, E. A. & Stannett, R. H. O. (1964). *Electron. Engng*, **36**, 159.
- Fritz, I. B. & Yue, K. T. N. (1963). *Advanc. Lipid Res.* **1**, 285.
- Garbus, J. & Weinbach, E. C. (1963). *Fed. Proc.* **22**, 405.
- Garland, P. B. (1964). Ph.D. Dissertation: University of Cambridge.
- Garland, P. B. & Randle, P. J. (1964). *Biochem. J.* **91**, 6c.
- Garland, P. B., Shepherd, D. & Yates, D. W. (1965). *Biochem. J.* **97**, 587.
- Garland, P. B., Shepherd, D. & Nicholls, D. G. (1966). In *Mitochondrial Structure and Compartmentation*. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Bari: Adriatica Editrice (in the Press).
- Garland, P. B., Shepherd, D., Nicholls, D. G., Yates, D. W. & Light, P. A. (1967). In *Control of the Citric Acid Cycle*. Ed. by Lowenstein, J. New York: Marcel Dekker Inc. (in the Press).
- Goebell, H. & Klingenberg, M. (1964). *Biochem. Z.* **340**, 441.
- Greville, G. D. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 86. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Gutfreund, H. (1965). *An Introduction to the Study of Enzymes*, p. 149. Oxford: Blackwell Scientific Publications.
- Haslam, R. J. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 108. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Hathaway, J. A. & Atkinson, D. E. (1963). *J. biol. Chem.* **238**, 2875.
- Hathaway, J. A. & Atkinson, D. E. (1965). *Biochem. biophys. Res. Commun.* **20**, 661.
- Henning, H.-V. & Seubert, W. (1964). *Biochem. Z.* **340**, 160.
- Hunter, F. E. (1951). In *Phosphorus Metabolism*, vol. 1, p. 297. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
- Kalkhoff, R. K., Hornbrook, K. R., Burch, H. B. & Kipnis, D. M. (1966). *Diabetes*, **15**, 451.
- Keech, D. B. & Utter, M. F. (1963). *J. biol. Chem.* **238**, 2609.
- Klingenberg, M. & Slenczka, W. (1959). *Biochem. Z.* **331**, 486.
- Krebs, H. A. (1964). *Proc. Roy. Soc. B*, **159**, 545.
- Lardy, H. A., Paetkau, V. & Walter, P. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1410.
- Matthews, M. D., Faulkner, E. A. & Green, G. W. (1965). *Electron. Engng*, **37**, 472.
- Meldrum, N. U. & Roughton, F. J. W. (1933). *J. Physiol.* **80**, 113.
- Morrison, J. F. & Peters, R. A. (1954). *Biochem. J.* **53**, 473.
- Mullard Ltd. (1963). *Reference Manual of Transistor Circuits*, p. 271. London: Mullard Ltd.
- Nicholls, D. G. & Garland, P. B. (1966). *Biochem. J.* **100**, 77p.
- Ochoa, S., Stern, I. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.
- Parmeggiani, P. & Bowman, R. H. (1963). *Biochem. biophys. Res. Commun.* **12**, 268.
- Sauer, F. & Erfle, J. D. (1966). *J. biol. Chem.* **241**, 30.
- Scholz, R. & Bücher, Th. (1965). In *Control of Energy Metabolism*, p. 393. Ed. by Chance, B., Estabrook, R. W. & Williamson, J. R. New York and London: Academic Press Inc.
- Seubert, W. (1960). *Biochem. Prep.* **7**, 80.
- Severinghaus, J. W. & Bradley, A. F. (1958). *J. appl. Physiol.* **13**, 515.
- Shepherd, D. & Garland, P. B. (1966). *Biochem. biophys. Res. Commun.* **22**, 89.
- Shepherd, D., Yates, D. W. & Garland, P. B. (1965). *Biochem. J.* **97**, 38c.
- Siebert, G. (1963). In *Methods of Enzymatic Analysis*, p. 318. Ed. by Bergmeyer, H.-U. London: Academic Press (Inc.) Ltd.
- Simon, E. J. & Shemin, D. (1953). *J. Amer. chem. Soc.* **75**, 2520.
- Stow, R. W., Baer, R. F. & Randall, G. F. (1957). *Arch. phys. Med.* **38**, 646.
- Struck, E., Ashmore, J. & Wieland, O. (1965). *Biochem. Z.* **343**, 107.
- Stuart, S. C. & Williams, G. R. (1967). *Biochemistry* (in the Press).
- Tubbs, P. K. & Garland, P. B. (1964). *Biochem. J.* **93**, 550.
- Utter, M. F. & Keech, D. B. (1963). *J. biol. Chem.* **238**, 2603.
- Utter, M. F., Keech, D. B. & Scrutton, M. C. (1964). In *Advances in Enzyme Regulation*, vol. 2, p. 49. Ed. by Weber, G. London: Pergamon Press Ltd.
- Walter, P., Paetkau, V. & Lardy, H. A. (1966). *J. biol. Chem.* **241**, 2523.
- Wieland, O. & Weiss, L. (1963). *Biochem. biophys. Res. Commun.* **10**, 333.
- Williams, G. R. (1965). *Canad. J. Biochem.* **43**, 603.
- Williamson, J. R. (1967). In *Advances in Enzyme Regulation*, vol. 5. Ed. by Weber, G. London: Pergamon Press Ltd. (in the Press).
- Zanwal, B. D., Zink, M. W. & Stachow, C. S. (1964). *J. biol. Chem.* **239**, 1597.