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Carnitine and Derivatives in Rat Tissues

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1. Free carnitine, acetylcarnitine, short-chain acylcarnitine and acid-insoluble carnitine (probably long-chain acylcarnitine) have been measured in rat tissues. 2. Starvation caused an increase in the proportion of carnitine that was acetylated in liver and kidney; at least in liver fat-feeding had the same effect, whereas a carbohydrate diet caused a very low acetylcarnitine content. 3. In heart, on the other hand, starvation did not cause an increase in the acetylcarnitine/carnitine ratio, whereas fat-feeding caused a decrease. The acetylcarnitine content of heart was diminished by alloxan-diabetes or a fatty diet, but not by re-feeding with carbohydrate. 4. Under conditions of increased fatty acid supply the acid-insoluble carnitine content was increased in heart, liver and kidney. 5. The acylation state of carnitine was capable of very rapid change. Concentrations of carnitine derivatives varied with different methods of obtaining tissue samples, and very little acid-insoluble carnitine was found in tissues of rats anaesthetized with Nembutal. In liver the acetylcarnitine (and acetyl-CoA) content decreased if freezing of tissue samples was delayed; in heart this caused an increase in acetylcarnitine. 6. Incubation of diaphragms with acetate or DL-β-hydroxybutyrate caused the acetylcarnitine content to become elevated. 7. Perfusion of hearts with fatty acids containing an even number of carbon atoms, DL-β-hydroxybutyrate or pyruvate resulted in increased contents of acetylcarnitine and acetyl-CoA. Accumulation of these acetyl compounds was prevented by the additional presence of propionate or pentanoate in the perfusion medium; this prevention was not due to extensive propionylation of CoA or carnitine. 8. Perfusion of hearts with palmitate caused a severalfold increase in the content of acid-insoluble carnitine; this increase did not occur when propionate was also present. 9. Comparison of the acetylation states of carnitine and CoA in perfused hearts suggests that the carnitine acetyltransferase reactants may remain near equilibrium despite wide variations in their steady-state concentrations. This is not the case with the citrate synthase reaction. It is suggested that the carnitine acetyltransferase system buffers the tissue content of acetyl-CoA against rapid changes.

The oxidation of long-chain fatty acids by mitochondrial preparations from various tissues was shown by Fritz (1955, 1959) to be stimulated by (−)-carnitine, and it has been suggested (Bremer, 1962*a*; Fritz & Yue, 1963) that long-chain acyl groups are transferred from an extramitochondrial site of activation to the intramitochondrial site of oxidation in the form of carnitine esters. Enzymes catalysing the reversible transfer of both long- and short-chain acyl groups between CoA and carnitine have been demonstrated in many tissues; a long-chain acyltransferase has been partly purified from calf liver by Norum (1964), and the short-chain acyltransferase from pigeon muscle has been crystallized (Chase, Pearson & Tubbs, 1965).

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It has been shown that in rat tissues marked alterations in the acylation state of CoA accompany metabolic changes (Wieland, Loeffler, Weiss & Neufeldt, 1960; Wieland & Weiss, 1963; Tubbs & Garland, 1963, 1964; Bortz & Lynen, 1963; Garland & Randle, 1964*a*,*b*). Analogous effects with carnitine have been briefly reported (Pearson & Tubbs, 1964*a*,*b*; Tubbs, Pearson & Chase, 1965); the present paper reports these in more detail.

MATERIALS

Albino rats each weighing 200–250g. were used for experiments *in vivo*; hearts and diaphragms from rats each weighing 250–300g. were used for experiments *in vitro*. Unless otherwise stated, the rats were given a balanced laboratory diet *ad libitum*. Starved animals were deprived of food for 36–48hr. Fat-re-fed rats were starved for 36hr.

and then given beef suet for 48hr. before being killed; carbohydrate-re-fed animals were similarly starved and then given bread and sucrose for 48hr. In some cases rats were rendered diabetic by an intravenous injection of alloxan (60mg./kg.) 48–50hr. before killing.

Materials were obtained from the following sources: ATP, NAD^+, NADH, CoA and malate and lactate dehydrogenases were from Boehringer Corp. (London) Ltd., London, W. 5, and DL-carnitine was from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Sorbic acid, from Kodak Ltd., London, was twice recrystallized from water; bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was purified by the method of Garland, Newsholme & Randle (1964). Insulin was from Wellcome Research Laboratories, Beckenham, Kent, heparin from Evans Medical Ltd., Liverpool and Nembutal from Abbott Laboratories Ltd., Queenborough, Kent.

Acetyl-DL-carnitine was prepared by the modification of the method of Fraenkel & Friedman (1957) described by Bremer (1962*a*), and palmitoyl-DL-carnitine by the method of Bremer (1962*b*). Acetyl-CoA was made by a modification of the method of Simon & Shemin (1953).

Citrate synthase (EC 4.1.3.7) was crystallized from pig heart (Srere & Kosicki, 1961), and carnitine acetyltransferase (EC 2.3.1.7) was purified from pigeon breast muscle (Chase *et al.* 1965). Acyl-CoA synthetase (EC 6.2.1.2) was prepared from ox-liver mitochondria by a modification of the method of Mahler, Wakil & Bock (1953). The fraction C of these authors was dialysed against two changes of 10mM-tris–chloride, pH 8·7, and calcium phosphate gel was then added (0·8mg./mg. of protein). This gel was removed by centrifuging, and to the supernatant was added more calcium phosphate (half the previous amount); this also was discarded. This step effectively removes all carnitine acetyltransferase. The protein in the supernatant was precipitated with $(NH_4)_2SO_4$ (50g. added/100ml. of solution), and dissolved in the above tris buffer to give a concentration of about 10mg./ml. After dialysis as above, the enzyme was applied to a column of DEAE-cellulose equilibrated with the tris buffer; the column was washed with about 3 bed-volumes of tris containing 40mM-KCl, and the synthetase was eluted with a similar volume of tris containing 80mM-KCl. After precipitation with $(NH_4)_2SO_4$ as above, the enzyme was dissolved in a small volume of 20mM-$KHCO_3$ and stored at −15°. The synthetase used catalysed the formation of 0·6–0·7 μmole of sorboyl-CoA/min./mg. at 25°.

METHODS

Experiments in vivo. In some cases tissues were removed from rats as quickly as possible after decapitation, and frozen in acetone containing solid CO_2. In other cases animals were anaesthetized with Nembutal (60mg./kg., injected intraperitoneally); tissues could then be removed and frozen before becoming markedly anaerobic, and liver and skeletal muscle (soleus) were frozen *in situ* by being clamped between aluminium blocks (diam. 2·5cm., thickness 1·3cm.) pre-cooled in liquid N_2 (Wollenberger, Ristau & Schoffa, 1960). Frozen tissue was powdered and extracted with $HClO_4$ as previously described (Pearson & Tubbs, 1964*a*,*b*). With epididymal fat pad, the frozen tissue was extracted with 3% (w/v) $HClO_4$–ether (1:1, v/v) and the aqueous phase was neutralized, freeze-dried and dissolved in water (0·5ml./g. of frozen tissue).

Experiments in vitro. Hemidiaphragms were obtained from decapitated male rats as described by Randle & Smith (1958). Pairs of hemidiaphragms were placed in 25ml. conical flasks containing 4ml. of bicarbonate medium (Krebs & Henseleit, 1932) with appropriate additions. After being gassed with O_2+CO_2 (95:5), the flasks were sealed and incubated with shaking at 37°. After 20min. the tissue was removed, quickly blotted, and frozen in acetone containing solid CO_2.

Ten min. before their hearts were removed for perfusion experiments, female rats were injected intraperitoneally with heparin (2000units/kg.) and Nembutal (60mg./kg.). The hearts were perfused at 37° with bicarbonate medium (Krebs & Henseleit, 1932) containing glucose (1mg./ml.) and insulin (0·02unit/ml.) and gassed with O_2+CO_2 (95:5) in the apparatus of Morgan, Henderson, Regen & Park (1961). Other substrates were added as mentioned below. Palmitate-containing medium was prepared by slowly injecting hot sodium palmitate solution into stirred Krebs–Henseleit bicarbonate containing 2% (w/v) defatted bovine serum albumin but lacking Ca^{2+}; this was then dialysed overnight against medium containing the usual amount of Ca^{2+}. Perfusion was usually by drip-through (Newsholme & Randle, 1961) for 10min. When albumin was present the heart was washed through with about 10ml. of medium, and then 15ml. was circulated for 8min. with a peristaltic pump adjusted to give a perfusion pressure of 40–50mm.Hg. At the end of perfusion the hearts were frozen by dropping them into acetone containing solid CO_2.

Assay of metabolic intermediates. In general, assays were carried out in a Beckman DK-2 recording spectrophotometer.

CoA was assayed with sorbate and acyl-CoA synthetase (Wakil & Hübscher, 1960), assuming an increase in molar extinction at 300mμ for the formation of sorboyl-CoA of 23×10^3cm.$^{-1}$ (P. K. Tubbs & D. J. Pearson, unpublished work). Carnitine was assayed by coupling the CoA assay to carnitine acetyltransferase as described by Pearson & Tubbs (1964*c*), except that GSH was omitted and the pH was 8·2. Acetyl-CoA was assayed by malate dehydrogenase and citrate synthase (Wieland & Weiss, 1963) in a system containing: tris–HCl, pH 8·0, 100mM; L-malate, 7·5mM; NAD^+, 0·4mM; and tissue extract. Malate dehydrogenase and citrate synthase were added consecutively, and a correction (Pearson, 1965) was applied to allow for the non-stoicheiometric formation of NADH. Short-chain acyl-CoA (including acetyl-CoA) was measured in an assay system (2ml.) containing: tissue extract, 1ml.; tris–HCl, pH 8·2, 100mM; ATP, 5mM; sorbate, 5mM; $MgCl_2$, 10mM; EDTA, 0·5mM; DL-carnitine, 10mM. The increase in extinction at 300mμ on adding acyl-CoA synthetase (about 0·7mg.) corresponded to the free CoA in the sample, and the acyl-CoA was determined by subsequent addition of carnitine acetyltransferase (about 10μg.). The acetyl-CoA content of the tissue extract, determined in the citrate synthase system described above, was subtracted to obtain the amount of other short-chain acyl-CoA derivatives; citrate synthase is specific for acetyl-CoA, and carnitine acetyltransferase is active with acyl groups shorter than decanoyl (Chase, 1967).

Acetylcarnitine was measured in the same assay system as acetyl-CoA, but with the additional constituents CoA (0·1mM) and EDTA (0·5mM). After completion of the coupled malate dehydrogenase and citrate synthase

reaction, carnitine acetyltransferase (10μg.) was added, and the acetylcarnitine concentration derived from the additional NADH formed. Short-chain acylcarnitine (including acetylcarnitine) was measured in an assay system containing, in a final vol. of 2ml.: tissue extract, 0·2–0·3ml.; tris–HCl, pH 8·0, 100mM; CoA, 0·2mM; EDTA, 0·3mM. The net increase in extinction at 232mμ after the addition of carnitine acetyltransferase (about 5μg.) was recorded, and the acylcarnitine concentration was derived assuming a molar extinction for acyl-CoA formation of $4{\cdot}5 \times 10^3$cm.$^{-1}$ (Stadtman, 1957) and an equilibrium constant of 0·6 for the transferase reaction (Fritz, Schultz & Srere, 1963). The concentration of free and acylated CoA and of free carnitine in the tissue extract are in principle needed for the calculation of the correction, but the amounts of the first two in the assay system are usually too low for significant interference. The acetylcarnitine content, determined by the coupled assay described above, was subtracted to obtain the amount of other short-chain carnitine esters.

Free (non-esterified) carnitine was taken to be the carnitine found when tissue extracts were examined without prior exposure to alkali. The total acid-soluble and acid-insoluble carnitine fractions were prepared for assay as previously described (Pearson & Tubbs, 1964*b*; Tubbs *et al.* 1965); treatment with *N*-ethylmaleimide (Pearson & Tubbs, 1964*c*) was omitted. For reasons previously cited (Pearson & Tubbs, 1964*b*) the acid-insoluble carnitine content is assumed to be long-chain esters; the acid-soluble fraction contains short-chain derivatives and free carnitine.

Citrate was assayed with citrate lyase from *Aerobacter aerogenes* (kindly given by Dr R. M. Denton), by a modification of the method of Dagley (1963). Pyruvate was determined by following the oxidation of excess of NADH at

Table 1. *Contents of carnitine and derivatives, and of acetyl-CoA, in rat tissues*

Tissue samples were removed immediately after rats (female in heart, liver and kidney experiments, male in other cases) had been killed by decapitation, or, where indicated, by Nembutal anaesthesia, and were frozen by being clamped between metal blocks cooled in liquid N_2 or by immersion in acetone containing solid CO_2. The frozen tissue was extracted and analysed as described in the text. Except where otherwise stated rats were given a standard laboratory diet *ad libitum* (controls); others were killed either after starvation for 36–48hr., or after starvation and subsequent re-feeding for 48hr. with fat (beef suet) or carbohydrate (bread and sucrose). Results are means ± S.E.M.; dashes indicate quantities not determined.

			Concns. (mμmoles/g. of frozen tissue)				
Tissue	Conditions	No. of rats	Free carnitine	Acetyl-carnitine	Acid-insoluble carnitine	Total acid-soluble carnitine	Acetyl-CoA
Heart	Control	7	302±46	377±62	52±23	779± 95	<5
	Starved	4	301±65	321±82	148±13	713± 87	<5
	Fat-re-fed	4	274±73	182±19	184±57	547±100	<5
	Carbohydrate-re-fed	4	477±25	384±38	20±14	857± 73	<5
	Alloxan-diabetic	4	169±48	204±59	202±42	534± 75	<5
	Control (Nembutal)	4	542±38	124±33	<5	837± 62	<5
	Starved (Nembutal)	4	530±92	145±44	11± 1	868± 35	<5
Liver	Control	5	173±24	41± 9	11± 1	296± 24	26 (2 only)
	Starved	6	130±28	95±16	76± 4	341± 30	49 (2 only)
	Fat-re-fed	4	140±15	61±11	67±23	278± 34	—
	Carbohydrate-re-fed	4	186±23	<10	6± 2	316± 30	—
	Control (Nembutal)	4	138±24	74±12	<10	255± 20	41±7
	Starved (Nembutal)	4	132±24	289±26	<10	426± 20	116±5
	Starved (ether)	2	57	332	30	423	124
Kidney	Control	5	310±47	93±17	16± 2	487± 50	18±4
	Starved	4	198±16	155±20	47± 8	456± 11	26±6
	Control (Nembutal)	3	284±30	120±22	<10	427± 11	24±3
	Starved (Nembutal)	3	99±11	301±28	<10	373± 3	43±2
Diaphragm	Control	3	285±21	207±55	—	464± 41	<5
Soleus muscle	Control	3	442±60	<50	—	627± 57	—
	Control (Nembutal)	3	483±48	<20	—	564± 25	—
Brain	Control	3	43± 6	<10	—	50± 6	—
Adrenal	Control	3	185±22	<20	—	262± 18	—
Testis	Control	3	91± 5	47± 8	—	186± 11	—
Epididymis	Control	2	6200	540	—	—	—
Epididymal fat pad	Control	4	10± 1	<2	—	16± 2	—

340mμ in the presence of lactate dehydrogenase, and lactate by the method of Hohorst, Kreutz & Bücher (1959).

RESULTS

Measurements 'in vivo'. Contents of carnitine compounds and of acetyl-CoA found in rat tissues '*in vivo*' (i.e. in rapidly frozen tissue samples) are given in Table 1. The total amount of carnitine in heart, liver and kidney was not markedly altered by starvation or changes of diet over the course of a few days. Apart from the special case of epididymis (see Marquis & Fritz, 1965*b*), heart and skeletal muscle contained the largest amounts of total carnitine, kidney and liver rather less, and brain very little. In contrast with the report of Abdel-Kader & Wolf (1965), only moderate amounts were found in adrenal gland. Samples of leg muscle (soleus) differed from heart, liver, kidney and diaphragm in that very little of the carnitine was acetylated; however, this may to some extent have been an artifact of isolation. In many cases the total acid-soluble carnitine appeared to exceed by an appreciable margin the sum of free carnitine and acetylcarnitine, although, as mentioned later, other short-chain acyl derivatives were not normally detectable; thus in one experiment the supernatant fraction of an acid extract of liver from anaesthetized starved rats, containing 40mμmoles of acetylcarnitine/ml. and 30mμmoles of free carnitine/ml., finally contained 87mμmoles of carnitine/ml. after exposure to 0·1N-potassium hydroxide at room temperature. All the acetylcarnitine had disappeared after 6min., but further carnitine continued to accumulate for another 6–7min. The original form of the extra carnitine is unknown.

In the heart, liver and kidney of decapitated animals, acid-insoluble carnitine concentrations were much higher in starved rats than in controls. High tissue contents of acid-insoluble carnitine were also observed in heart and liver in fat-re-fed animals, and in heart in alloxan-diabetes; in carbohydrate-re-fed rats, on the other hand, concentrations of insoluble carnitine were very low.

Starvation resulted in an increase in the acetylcarnitine content at the expense of free carnitine in liver and kidney. In liver (kidney was not tested) fat-re-feeding produced the same effect, whereas carbohydrate feeding resulted in a very low acetylcarnitine content. It is well established (Wieland & Weiss, 1963; Tubbs & Garland, 1964) that, in liver, acetyl-CoA shows comparable fluctuation. In heart, the behaviour of acetylcarnitine was completely different; starvation did not cause an increase in amount, and re-feeding experiments showed that fat caused a fall, and carbohydrate a rise, in the concentration. Garland & Randle (1964*b*) have previously reported that starvation does not cause an increase in the acetyl-CoA content of rat heart.

Tissues from rats anaesthetized with Nembutal or ether gave results different from those obtained from decapitated animals. Carnitine was less acetylated in heart from anaesthetized animals, but the opposite was found with liver and kidney. The content of acid-insoluble carnitine was very low in liver, kidney and heart from Nembutal-anaesthetized animals, even when these had been starved. With rats that had been anaesthetized with ether the results were similar to those observed with Nembutal, although rather larger amounts of acid-insoluble carnitine were found (up to 40mμmoles/g. in the hearts of starved animals). Tissue samples from anaesthetized rats were much less anaerobic than those obtained after decapitation (for heart, lactate/pyruvate ratios were about 25–40 and 80–150 respectively).

At least in liver and kidney, acetyl-CoA resembled acetylcarnitine in that the concentrations were increased by anaesthesia as compared with decapitation, whereas, as with acid-insoluble carnitine, the opposite was found with fatty acyl-CoA.

Effect of ischaemia on acetyl-CoA and acetylcarnitine content. Portions of liver were obtained from starved rats anaesthetized with Nembutal, and were frozen at various times after removal. Zero-time samples were frozen *in situ* by means of aluminium blocks pre-cooled in liquid nitrogen. Fig. 1 shows the concentrations of free and acetylated carnitine and of acetyl-CoA after various delays before freezing.

A rapid decrease in acetyl-CoA content was accompanied by a similar decrease in acetylcarnitine content and a corresponding increase in free carnitine. Fig. 1 also shows the very rapid disappearance of acetyl-CoA that would have occurred in the absence of carnitine acetyltransferase activity, on the assumption that acetyl-CoA was the sole initial product of acetylcarnitine removal. This assumption is probably justified, since attempts to detect enzymes (e.g. hydrolases) acting on acetylcarnitine have all been unsuccessful. No short-chain acylcarnitine other than the acetyl ester was detected in the experiment shown in Fig. 1.

In hearts, contrary to the observations with liver, the content of acetylcarnitine increased if freezing was delayed (in hearts from ether-anaesthetized rats, fed *ad libitum*, an initial acetylcarnitine content of about 100mμmoles/g. wet wt. increased to about 250mμmoles/g. after 40sec. delay). This increase agrees with the observation (Table 1) that rapidly frozen (but almost anaerobic) hearts from decapitated animals contained more acetylcarnitine than hearts obtained after anaesthesia, and with the report by Garland & Randle

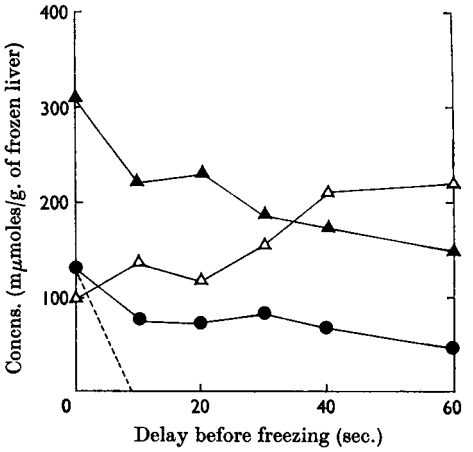

Fig. 1. Changes in rat liver contents of acetyl-CoA, acetylcarnitine and free carnitine as a function of delay between excision of tissue samples and freezing. Liver samples were removed from starved female rats anaesthetized with Nembutal, and rapidly frozen (Wollenberger *et al.* 1960) after the delays shown. Zero-time samples were frozen *in situ*. Analyses were carried out as described in the text. ●, Acetyl-CoA; ▲, acetylcarnitine; △, free carnitine. The broken line shows the disappearance of acetyl-CoA expected if regeneration from acetylcarnitine did not occur.

Table 2. *Contents of carnitine in isolated rat hemidiaphragms*

Hemidiaphragms (Randle & Smith, 1958) from decapitated male rats were incubated in bicarbonate medium (Krebs & Henseleit, 1932) containing glucose (1·5mg./ml.), insulin (0·02unit/ml.) and other additions as shown. After 20min. at 37° the tissue was frozen and analysed; for details see the text. Results are the means ± S.E.M.

Additions to glucose medium	No. of samples	Concns. (mμmoles/g. of frozen diaphragm) Free carnitine	Acetylcarnitine	Total acid-soluble carnitine
None	3	369±21	67±11	501±23
Acetate (4mM)	2	253	180	543
DL-β-Hydroxybutyrate (5·5mM)	3	255±21	169±40	509±52

(1964*b*) that measured acetyl-CoA amounts in rat heart are lowered when freezing is very rapid.

Measurements in vitro. (*a*) Incubated diaphragms. Table 2 shows that the addition of acetate or DL-β-hydroxybutyrate to a glucose medium caused increased acetylation of carnitine in isolated diaphragms.

(*b*) Perfused hearts. The acylation state of CoA in perfused hearts is greatly affected by perfusion conditions (Garland & Randle, 1964*a*,*b*; Bowman, 1966), and, as has been briefly reported before (Pearson & Tubbs, 1964*a*,*b*; Tubbs *et al.* 1965), the same is true for carnitine. Tables 3 and 4 show the effects of various additions to the perfusion medium.

The content of acetylcarnitine was greatly increased by perfusion with fatty acids containing an even number of carbon atoms, DL-β-hydroxybutyrate or pyruvate, but reduced by propionate and glutamate. The free carnitine content was decreased by perfusion with fatty acids, β-hydroxybutyrate or pyruvate and increased by glutamate. Short-chain acylcarnitine, other than acetylcarnitine, accumulated when the medium contained 4mM-propionate or α-oxobutyrate, and probably also with butyrate and β-hydroxybutyrate, although this was not directly measured. Acid-insoluble carnitine was elevated when palmitate was added, or when hearts from starved rats were used.

Table 1 shows that fat-feeding or alloxan-diabetes lowered the acetylcarnitine content of heart, and perfused hearts from starved rats contained less acetylcarnitine then those from fed controls (Table 3); however, perfusion with fatty acids or β-hydroxybutyrate raised the acetylcarnitine concentration. [The finding (Pearson & Tubbs, 1964*a*) that hearts from alloxan-diabetic rats contained high acetylcarnitine concentrations, even after perfusion with glucose and insulin, was not repeated in later work.] Garland *et al.* (1964) found that alloxan-diabetes or starvation lowered the glycerol phosphate/dihydroxyacetone phosphate ratio (and hence presumably the cytoplasmic NADH/NAD$^+$ ratio), whereas fatty acid perfusion raised it; these reports fit in with the observation that in heart (unlike liver) anoxia causes elevation of acetylcarnitine. The interrelationship and explanation of these contrary effects of conditions *in vivo* and during perfusion are not understood.

As expected, perfusion with fatty acids with an even number of carbon atoms, β-hydroxybutyrate or pyruvate, resulted in an increased acetyl-CoA content, and, where measured, a decrease in free CoA.

Perfusion of hearts under anaerobic conditions (medium gassed with nitrogen + carbon dioxide, 95:5) and the standard medium gave results similar to those of aerobic controls. The reason for the different effects of anaerobic perfusion and delayed freezing (see above) is not obvious.

As shown in Table 3, addition of 4mM-propionate or α-oxobutyrate to the standard glucose perfusion medium resulted in very low contents of free carnitine and acetylcarnitine, whereas short-chain acylcarnitine (presumably the propionyl derivative) accumulated. In hearts perfused with

Table 3. *Effects of substrates on the acylation states of carnitine and CoA in perfused rat hearts*

Hearts were perfused at 37° for 10min. with bicarbonate medium (Krebs & Henseleit, 1932) containing glucose (1mg./ml.), insulin (0·02unit/ml.) and other additions as shown. The hearts were then frozen and analysed. Other details are given in the text. Results are means ± S.E.M.; dashes indicate quantities not determined. The free CoA contents in square brackets are taken from Garland & Randle (1964*b*).

		Concns. (mμmoles/g. of frozen heart)						
Additions to standard perfusion medium	No. of hearts	Free carnitine	Acetyl-carnitine	Short-chain acyl-carnitine (including acetyl-carnitine)	Total acid-soluble carnitine	Acid-insoluble carnitine	Free CoA	Acetyl-CoA
None	10	590±51	90±40	—	783±83	17 (2 only)	69 (2 only)	<5
None (starved rats)	3	587±63	44±26	—	735±40	45± 7	[67±2]	<5
Acetate (4mM)	11	257±50	439±49	—	836±49	—	24 (2 only)	35± 7
Propionate (4mM)	5	62±16	<10	670 (2 only)	801±55	—	10 (3 only)	<5
Butyrate (4mM)	3	123±11	418±38	—	890±34	—	[28±4]	33± 2
DL-β-Hydroxybutyrate (1·4mM)	2	286	325	—	833	—	—	16
DL-β-Hydroxybutyrate (5·5mM)	10	211±15	513±57	610 (2 only)	845±44	—	[30±4]	41±11
DL-β-Hydroxybutyrate (5·5mM), no glucose or insulin	3	115±13	582±35	—	795±27	—	25±5	63± 4
Albumin (2%, w/v)	6	592±57	153±63	120 (2 only)	—	22±4	[63±5]	<5
Albumin + palmitate (0·75mM)	6	308±54	409±41	480 (2 only)	—	111±32	[29±3]	19± 3
Pyruvate (4mM)	4	401±16	414±72	—	906±82	—	—	16± 2
α-Oxobutyrate (4mM)	3	60±17	12± 1	—	798±64	—	—	<5
L-Glutamate (4mM)	3	803±31	43±11	—	942±45	—	—	<5

4mM-propionate, short-chain acyl-CoA was found (42 ± 3mμmoles/g., three hearts), although the acetyl-CoA concentration was less than 5mμmoles/g.

It was decided to investigate the effects of these compounds and another possible propionyl-CoA precursor, *n*-pentanoate, on the elevation of acetylcarnitine caused by acetate and appropriate other substrates (Table 3). Table 4 shows the results of such experiments.

It is evident that propionate and pentanoate, and to a lesser degree α-oxobutyrate, were effective in overcoming the acetylation of carnitine and CoA caused by perfusion with acetate. Indeed, although equimolar amounts of acetyl-CoA and propionyl-CoA presumably arise from the catabolism of pentanoate this acid was even more effective than propionate in preventing acetylation by acetate.

Propionate was also highly effective in preventing accumulation of acetylcarnitine and acetyl-CoA in hearts perfused with β-hydroxybutyrate, pyruvate or palmitate; this indicates that acetyl-CoA synthetase was not the locus of propionate (and presumably pentanoate) action.

Table 4, as well as Table 3, shows that when the perfusion medium contained propionate (in addition to β-hydroxybutyrate or palmitate) the accumulation of propionylcarnitine could be demonstrated directly (the acylcarnitine could not have been the methylmalonyl or succinyl derivatives, since the enzymic assay is specific for monocarboxylic esters of carnitine). Short-chain acylcarnitine accumulation obviously also occurred when pentanoate was present, and Table 3 shows this to be true with α-oxobutyrate as well. The possibility thus arose that these acids might prevent acetylcarnitine or acetyl-CoA formation by effectively trapping free carnitine or CoA as the propionyl derivatives, and Tables 3 and 4 show that the concentration of free carnitine can in fact be lowered by their presence in perfusion experiments. At least with propionate this was also the case with CoA. However, Table 4 also shows that the addition of 1·5mM-propionate to a medium containing 4mM-acetate caused an increase in free carnitine, although it nevertheless prevented formation of acetyl compounds.

Fig. 2 shows the results of an experiment in which rat hearts were perfused with the standard glucose medium containing 4mM-acetate and various concentrations of propionate; the average contents of free and acetylated forms of carnitine and CoA in hearts perfused in the absence of acetate (Table 3) are also indicated. A very low concentration of

Table 4. *Effects of propionyl-CoA precursors on the metabolite contents of rat hearts perfused with acetate, DL-β-hydroxybutyrate, pyruvate or palmitate*

Hearts were perfused with glucose–insulin medium as described in Table 3 and the text, except that other substrates were added as shown. The hearts were then frozen and analysed. Results are means ± S.E.M.; dashes indicate quantities not determined. The citrate content in square brackets is taken from Garland & Randle (1964*b*).

		Concns. (mμmoles/g. of frozen heart)					
Addition to standard perfusion medium	No. of hearts	Free carnitine	Acetyl-carnitine	Short-chain acyl-carnitine (including acetyl-carnitine)	Acid-insoluble carnitine	Acetyl-CoA	Citrate
None	10	590±51	90±40	—	17 (2 only)	<5	[220±20]
Acetate (4mM)	11	257±50	439±49	—	—	35± 7	522±60 (4 only)
Acetate+propionate (1·5mM)	7	450±32	37±12	—	—	<5	660 (2 only)
Acetate+pentanoate (0·4mM)	3	130±10	33± 9	—	—	<5	532±80 (3 only)
Acetate+pentanoate (1·5mM)	3	120±13	<10	—	—	<5	226±66 (3 only)
Acetate+α-oxobutyrate (1·5mM)	3	282±37	323±57	—	—	17± 4	—
DL-β-Hydroxybutyrate (5·5mM)	10	211±15	513±57	610 (2 only)	—	41±11	590 (2 only)
DL-β-Hydroxybutyrate+propionate (1·5mM)	3	197±13	20± 5	587±4	—	<5	532±75 (3 only)
Pyruvate (4mM)	4	401±16	414±72	—	—	16± 2	—
Pyruvate+propionate (1·5mM)	2	202	17	—	—	<5	—
Albumin (2%)+palmitate (0·75mM)	6	308±54	409±41	480 (2 only)	111±32	19± 3	414 (2 only)
Albumin+palmitate+propionate (1·5mM)	2	34	<10	790	20	<5	404 (2 only)

propionate (0·4mM) was found to counteract completely the effects of 4mM-acetate on the acetylation state of carnitine and CoA, in that acetylation was almost completely prevented and very little of the carnitine and CoA was propionylated. As previously observed, the presence of higher propionate concentrations resulted in decreased contents of free carnitine and CoA, accompanied by accumulation of the propionyl esters (it is possible that some of the 'free' CoA in these experiments was succinyl-CoA, which is labile under the assay conditions; however, the results suggest that the amounts of this were small, at least when high propionate concentrations were used). The experiment of Fig. 2 thus shows that sequestration of a major part of the tissue carnitine or CoA is not the mechanism by which propionate (and presumably pentanoate or α-oxobutyrate) prevents any extensive acetylation of these acyl acceptors.

Rat hearts can form succinate from propionate (Flavin, Ortiz & Ochoa, 1955), and so propionyl-CoA should be an oxaloacetate precursor. Rapid formation of oxaloacetate in this way could conceivably accelerate the synthesis of citrate from acetyl-CoA, and so prevent acetylcarnitine and acetyl-CoA accumulation. The oxaloacetate contents of hearts were not measured in this work, but Table 4 shows that the elevated citrate contents caused by acetate perfusion (see Garland & Randle, 1964*b*) were not appreciably affected by amounts of propionate or pentanoate that prevented acetylation of carnitine and CoA (although 1·5mM-pentanoate did cause a reduction in citrate concentration). Newsholme & Randle (1964) found that addition of 4mM-propionate to glucose–insulin perfusion medium, which (Table 3) causes disappearance of acetylcarnitine, did not affect the hexose 6-phosphate/fructose 1,6-diphosphate ratio in rat hearts; this implies no marked increase in citrate content. Addition of 1·5mM-α-oxoglutarate or L-aspartate to acetate-containing perfusion media had no effect on the acetylation state of carnitine and CoA, although these compounds might be expected to increase the oxaloacetate concentration [few data are available on the

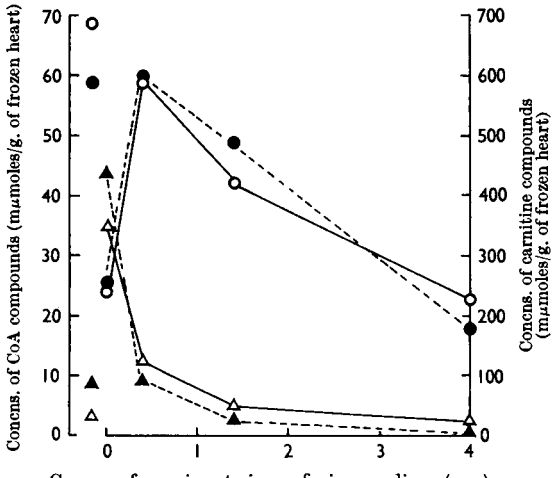

Fig. 2. Effect of propionate on the acetylation states of carnitine and CoA in rat hearts perfused with acetate. Hearts were perfused with a bicarbonate medium containing glucose (1mg./ml.), insulin (0·02unit/ml.), acetate (4mM) and propionate (0–4mM); they were then rapidly frozen and analysed. Details are given in the text. ●, Free carnitine; ▲, acetylcarnitine; ○, free CoA; △, acetyl-CoA. The disconnected points to the left show the situation when neither acetate nor propionate was present (see Table 3).

utilization by perfused hearts of oxoglutarate or aspartate, and it is possible that they penetrate poorly (Hicks & Kerly, 1960), although Bowman (1966) has shown that [U-^{14}C]aspartate is incorporated into malate and citrate by perfused rat hearts]. It seems unlikely therefore that the effects of propionate, pentanoate or α-oxobutyrate on the acetylation of CoA and carnitine can be explained on the simple basis that these are precursors of oxaloacetate and so assist removal of acetyl-CoA.

DISCUSSION

In heart, liver and kidney a small and variable proportion of the total carnitine was present in a perchloric acid-insoluble fraction (Tables 1, 3 and 4), from which it could be liberated by alkaline hydrolysis. For reasons previously given (Pearson & Tubbs, 1964*b*; Tubbs *et al.* 1965) this is assumed to be long-chain acylcarnitine. As reported in the earlier communications, and more recently by Bøhmer, Norum & Bremer (1966), the amount of such carnitine was increased under conditions of elevated free fatty acid concentration.

Of the carnitine extracted from frozen tissues by dilute perchloric acid, part only was free carnitine, many tissues containing considerable amounts of acetylcarnitine. Different dietary treatments, although not affecting the total content of carnitine, caused marked changes in its acylation state in heart, liver and kidney over the course of a few days. Since the observed acylation state varied with the method of killing rats, such changes can clearly take place very rapidly, and indeed the acetylcarnitine content of both liver and heart changed markedly during a few seconds of ischaemia [the low activities of rat liver carnitine acetyltransferase reported by Beenakkers & Klingenberg (1964) and Marquis & Fritz (1965*a*) are sufficient to account for the observed changes; see also Norum & Bremer (1967), who report a higher activity in rat liver].

The differences between samples from anaesthetized (comparatively aerobic) and decapitated (anaerobic) rats are in accord with the findings, reported above, that in ischaemia acetylcarnitine rises in heart but falls in liver. Dale (1962*a*,*b*) found ether anaesthesia to be less stressful than decapitation, and it may be that the relatively high content of acid-insoluble carnitine found in tissues from decapitated rats was a consequence of catecholamine-induced fatty acid mobilization (Gordon & Cherkes, 1958; Havel & Goldfein, 1959). Hales & Kennedy (1964) found a plasma free fatty acid concentration of 0·68mM in Nembutal-anaesthetized starved rats, and the data of Table 3 suggest that perfusion with this concentration of palmitate would cause high acid-insoluble carnitine levels; however, Havel & Goldfein (1959) found that deep anaesthesia lowered the plasma fatty acid concentration, and this may be the reason for the low contents of acid-insoluble carnitine found in samples from anaesthetized rats *in vivo*.

Alloxan-diabetes, like starvation or fat-feeding, lowered the acetylcarnitine content of heart despite the raising of the acid-insoluble (i.e. fatty acylcarnitine) fraction.

The results reported here agree quite well, at least qualitatively, with those of Bøhmer *et al.* (1966), who used decapitated rats and a completely different assay procedure in which tissue enzymes were inactivated by homogenization in chloroform–methanol rather than by rapid freezing. In particular, these workers also found that starvation, fat-feeding and diabetes all caused a decrease in the acetylcarnitine content of heart, although the fatty acylcarnitine content was raised. Bøhmer *et al.* (1966), however, did not observe the marked changes in liver acetylcarnitine that we have found, which are in accord with changes in acetyl-CoA content.

In all cases where acetyl-CoA was estimated in tissues, changes in the concentration of this compound were found to correspond to changes in acetylcarnitine content, irrespective of whether the changes were produced by alterations in diet,

sampling technique or perfusion conditions. Similarly, changes of free CoA in heart were accompanied by corresponding alterations of free carnitine content.

The ratio of acetylcarnitine to acetyl-CoA '*in vivo*' varies from tissue to tissue, being very high (over 20) in heart and diaphragm, considerably lower (5–10) in kidney, and least in liver (about 2); this largely reflects the total carnitine/total CoA ratio. Since no enzyme (other than carnitine acetyltransferase) has been found that forms or destroys acetylcarnitine, this compound may be a metabolic backwater; in this case it might be expected that the carnitine acetyltransferase equilibrium (Fritz *et al.* 1963):

$$K = \frac{[\text{Acetyl-CoA}]\cdot[\text{carnitine}]}{[\text{CoA}]\cdot[\text{acetylcarnitine}]} = 0{\cdot}6$$

would be maintained despite different steady-state concentrations of the reactants. Contents of free CoA have not often been measured, but Fig. 3 shows that under different perfusion conditions the acetyl-CoA/CoA or acetylcarnitine/carnitine ratios varied by more than 50-fold, and yet, within the considerable experimental error, the transferase reaction remained near equilibrium. With the very sensitive methods now developed (Pearson, Chase & Tubbs, 1968; Tubbs & Garland, 1968) for estimating the four reactants it should be possible to investigate this question with greater precision. The fact that the apparent equilibrium constant in perfused hearts remains near to that found with isolated enzyme implies that most of the CoA of heart is accessible to the transferase. Since this enzyme is very active in heart it would appear that acetylcarnitine must act as an acetyl-CoA buffer, in much the same way as high concentrations of malate and aspartate buffer the very small and rapidly turning-over pool of oxaloacetate, or creatine phosphate and other phosphagens damp charges in ATP content. Fabel & Lubbers (1965) have found the oxygen uptake of heart to remain constant throughout the contraction cycle, despite the variation in metabolic load, and remark that 'obviously the heart is very well buffered by energy-rich components'.

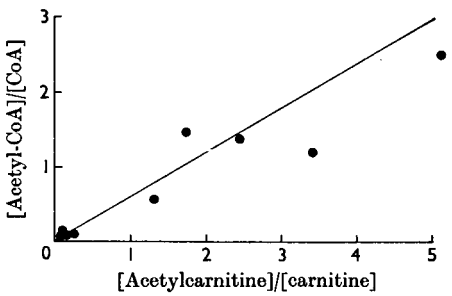

Fig. 3. Correlation between the acetylation states of carnitine and CoA in perfused rat hearts. Hearts were perfused with various substrate combinations that caused the acetylation states of carnitine and CoA to vary over a wide range (data in Tables 3 and 4, and Fig. 2). The line shows the expected correlation between the acetylation states of the two compounds on the assumption that the carnitine acetyltransferase is in equilibrium, so that:

$$\frac{[\text{Acetyl-CoA}]}{[\text{CoA}]} = 0{\cdot}6 \times \frac{[\text{Acetylcarnitine}]}{[\text{Carnitine}]}$$

(Fritz *et al.* 1963).

In liver, where the acetylcarnitine/acetyl-CoA ratio is lower than in heart and there is also less carnitine acetyltransferase, the buffering effect of carnitine would be expected to be less efficient; on the other hand the requirements for such an effect might be less demanding. Fig. 1 shows that in ischaemic rat liver the acetyl-CoA content would, in the absence of the carnitine acetyltransferase system, have diminished to zero in less than 10sec., whereas in fact considerable acetyl-CoA remained after 1min.; thus even in this tissue acetylcarnitine may function as an important short-time acetyl reservoir.

The acetyl-CoA buffering effect of acetylcarnitine proposed here and earlier (Tubbs *et al.* 1965) does not imply that this compound acts as a store in any long-term sense, since the amount present is so small that its combustion could only maintain respiration in rat heart or liver for a few seconds at most.

The maintenance of near-equilibrium in the carnitine acetyltransferase reaction contrasts sharply with the situation in the citrate synthase reaction, which will therefore not show simple mass-action responses and will be unable to buffer the acetyl-CoA/CoA ratio. From the data of Stern, Ochoa & Lynen (1952) it may be calculated that, at equilibrium at pH7·2:

$$K = \frac{[\text{Acetyl-CoA}]\cdot[\text{oxaloacetate}]}{[\text{Citrate}]\cdot[\text{CoA}]} = 2{\cdot}15 \times 10^{-6}$$

However, values for this 'constant' of between 2×10^{-3} and 20×10^{-3} may be calculated from the data for rat hearts reported by Garland & Randle (1964*b*) [perfused with glucose plus insulin and glucose plus insulin plus 5·5mM-DL-β-hydroxybutyrate respectively, with allowance (Pearson, 1965) for underestimation of acetyl-CoA]. In liver, also, the theoretical equilibrium constant is much exceeded. It is most unlikely that the discrepancy can be fully accounted for by experimental errors, or by the compartmentation of oxaloacetate (Tager, 1966) and citrate (Max & Purvis, 1965). This implies that the formation of citrate is exergonic in the steady state *in vivo*, and that citrate synthase

may indeed be a metabolic control point; the inhibition of this enzyme by fatty acyl-CoA (Tubbs, 1963; Wieland, Weiss & Eger-Neufeldt, 1964) and ATP (Hathaway & Atkinson, 1965; Shepherd & Garland, 1966) has been reported.

The buffer role of carnitine acetyltransferase in general fits in well with the tissue distribution of the enzyme (Beenakkers & Klingenberg, 1964), which correlates with high metabolic activity; the presence of extremely large amounts of the enzyme in spermatozoa and of carnitine in seminal plasma (Marquis & Fritz, 1965*b*) probably reflects a special metabolic function, whereas the absence of the transferase (Beenakkers & Klingenberg, 1964) or carnitine itself (Childress, Sacktor & Traynor, 1967) from the very active flight muscle of the bee suggests that this tissue may possess some alternative mechanism for damping rapid changes in acetyl-CoA content. It seems unlikely that even a relatively large pool of citric acid-cycle intermediates, as suggested by Childress *et al.* (1967), would be a suitable buffer. The foregoing discussion does not, of course, deny that in some organs, such as heart, or the liver of guinea pigs (Bressler & Katz, 1965) or ruminants, acetylcarnitine may be concerned in fatty acid synthesis; its implication in ketogenesis has also been proposed (Ontko, 1967).

The mechanism of the remarkable effect of propionate or pentanoate (Table 4 and Fig. 1) in preventing acetyl-CoA and acetylcarnitine accumulation, which might in some circumstances have important metabolic consequences, is obscure. As reported above, acetyl-CoA synthetase inhibition cannot be responsible. A straightforward competition for carnitine acetyltransferase is also ruled out, since propionyl-CoA does not have a particularly high affinity for the enzyme (Chase, 1967) and the anti-acetylation effect occurs when very little CoA or carnitine have become propionylated (Fig. 2). For reasons already given it also seems unlikely that rapid oxaloacetate formation, and subsequent enhancement of citrate formation and catabolism, i.e. acceleration of acetyl-CoA oxidation, is the explanation. It is noteworthy that propionate is capable of almost completely inhibiting the oxidation of acetate by slices of sheep or rat liver even when the acetate is in 10-fold excess (Masoro, Felts, Paragos & Rapport, 1957; Pennington & Appleton, 1958); Davis, Brown & Staubus (1960), however, observed no such effect with intact cattle. Leng & Annison (1963) reported that, conversely, acetate did not inhibit propionate oxidation by sheep liver. Pennington & Appleton (1958) observed that propionate prevents the formation of ketone bodies from pyruvate by sheep liver, although it did not stop pyruvate oxidation. Lang & Bassler (1953) found that propionate prevented the formation of acetoacetate from acetate by kidney mitochondria, even though these were incapable of oxidizing propionate.

A possible partial explanation for the effect of propionyl-CoA (i.e. propionate or pentanoate) in perfused rat heart might be that this blocks the transfer of acetyl-CoA between two (presumably mitochondrial) pools, designated (*a*) and (*b*) in Scheme 1.

Acetyl-CoA pool (*b*) is imagined to be capable of being much larger than pool (*a*). This Scheme accounts for the observations with liver and kidney mentioned above, and also perhaps for the fact that, although α-oxobutyrate can almost certainly give rise to propionyl-CoA in perfused hearts (Table 3), it is much less effective than propionate in cancelling the effect of acetate (Table 4); this might be because α-oxobutyrate gives propionyl-CoA in pool (*a*) whereas propionate and pentanoate form it in a more effective position (? pool *b*). The Scheme does not, however, satisfactorily account for the failure to prevent acetate-induced citrate accumulation in perfused hearts (Table 4). Even if such a speculative Scheme is in principle correct no explanation is offered for the actual mechanism by which a very small amount of propionyl-CoA exerts so marked an effect. The matter clearly merits further investigation with cell-free systems, since it may throw light on details of intramitochondrial organization of fatty acid metabolism.

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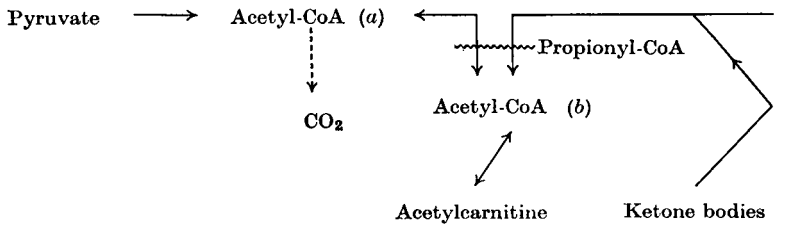

Scheme 1.

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