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# Variations in Tissue Contents of Coenzyme A Thio Esters and Possible Metabolic Implications

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Coenzyme A is involved in the metabolism of both carbohydrates and fatty acids. It is well established that these two aspects of cellular metabolism are closely interrelated; for example, ready availability of carbohydrate leads to rapid rates of fatty acid synthesis and esterification, whereas conversely high fatty acid concentrations spare the oxidation of glucose and favour gluconeogenesis. Carbohydrate and fatty acid catabolism both yield acetyl-coenzyme A, whereas fatty acids take part in very few biological reactions without prior formation of their coenzyme A thio esters. In view of the central metabolic role of coenzyme A it was thought profitable to investigate the contents of its derivatives *in vivo* in various circumstances, and to consider the metabolic implications of any variations found.

More specifically, interest in two problems led to this work. The first was the profound effect of dietary and other conditions on the rate of fatty acid synthesis in tissues (see Fritz, 1961; Masoro, 1962). For example, Hill, Webster, Linazasoro & Chaikoff (1960) found that oral administration of fat to rats 1–2 hr. before killing greatly impaired fatty acid synthesis in liver slices. The possibility that an intracellular inhibitor of fatty acid synthesis might be found was strengthened by the finding of Korchak & Masoro (1962) that, after starvation, fatty acid synthesis in liver slices was much more reduced than were the activities of the relevant enzymes as measured in dilute cell-free extracts. It was decided to investigate the possibility that the lipogenic inhibitor, perhaps formed by the liver microsomal fraction (Masoro & Porter, 1960), was in fact long-chain acyl-coenzyme A.

The second approach was an investigation of the possibility that the increased rate of fatty acid oxidation in tissues of starved and diabetic animals, usually attributed to an excessive fatty acid supply (see Fritz, 1961), is associated with an elevated intracellular content of long-chain acyl-coenzyme A.

Preliminary reports of parts of this work have been presented elsewhere (Tubbs & Garland, 1963; Garland & Tubbs, 1963; Tubbs, 1963).

## MATERIALS

*Rats.* Male albino Wistar rats of 200–300 g. body wt. were used, maintained except where otherwise stated on a balanced diet. Diabetes was induced by intravenous injection of alloxan (60 mg./kg.) under ether anaesthesia; the animals were killed 48–52 hr. later.

*Enzymes.* Acyl-CoA synthetase (EC 6.2.1.2) was prepared from ox-liver mitochondria by the method of Mahler, Wakil & Bock (1953). The enzyme was used either at the stage described by these authors as fraction C, or was further purified with diethylaminoethylcellulose and calcium phosphate gel. The enzyme was stored at pH 7·5 in 1·5M-$(NH_4)_2SO_4$ at 4°.

Citrate synthase (EC 4.1.3.7; citrate condensing enzyme) was prepared from pig heart by the method of Srere & Kosicki (1961*a*) and recrystallized twice. 'Fatty acid synthase' (the enzyme complex which synthesizes fatty acids from acetyl-CoA and malonyl-CoA) was partially purified from the livers of rats fed with a balanced diet *ad lib.* The liver was homogenized with 3 vol. of 0·1M-phosphate buffer, pH 6·6, at 0°, and the homogenate centrifuged for 1 hr. at 100000*g*. Solid $(NH_4)_2SO_4$ was added to the supernatant (24 g./100 ml.), and the precipitated protein dissolved in 0·05M-phosphate buffer, pH 6·6. The enzyme was used the same day as prepared.

Citrate ATP lyase (citrate cleavage enzyme) was obtained from the supernatant after centrifuging a homogenate of rat liver (20%, w/v, prepared in 0·25M-sucrose) at 100000*g* for 1 hr. The rats were starved for 24–48 hr. and then fed with bread for a similar period before killing.

Acyl-CoA dehydrogenase (EC 1.3.2.2) was partially purified as a by-product of the preparation of acyl-CoA synthetase, and assayed as described below with an initial palmitoyl-CoA concentration of 4 $\mu$M. In the preparation of acyl-CoA synthetase, fraction B described by Mahler *et al.* (1953) was the precipitate obtained from 55% saturated $(NH_4)_2SO_4$, and the supernatant contained acyl-CoA-dehydrogenase activity, which was precipitated by increasing the $(NH_4)_2SO_4$ saturation to 75%. This precipitate was redissolved in 0·02M-$KHCO_3$ to obtain a protein concentration of 10 mg./ml., and refractionated with solid $(NH_4)_2SO_4$ to obtain fractions precipitating at 40, 50 and 65% saturation. The fraction precipitating between 50 and 65% saturation was redissolved in a minimal volume of 0·02M-$KHCO_3$, and contained $8 \times 10^{-3}$ international unit of acyl-CoA-dehydrogenase activity/mg. of protein.

L-Malate dehydrogenase (EC 1.1.1.37), kindly given by Dr C. J. R. Thorne, was the mitochondrial isoenzyme from pig heart.

*Chemicals.* CoA was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, or from Farmochimica Cutolo-Calosi S.p.A., Naples, Italy. Acetyl- and butyryl-CoA were synthesized by the method of Simon & Shemin (1953). Malonyl-CoA was prepared by the method of Trams & Brady (1960). Palmitoyl-CoA was prepared either enzymically by the method of Kornberg & Pricer (1953), modified by the substitution of glycylglycine (pH 7·6) and 2-mercaptoethanol for phosphate and cysteine in the incubation mixture, or synthetically by the method of Seubert (1960).

$NAD^+$, NADH, NADPH, ATP and cytochrome *c* were obtained from C. F. Boehringer und Soehne, and phenazine methosulphate from the Sigma Chemical Co., St Louis, Mo., U.S.A. Palmitoyl-DL-carnitine was a gift from Mr D. J. Pearson. Sorbic acid (Eastman Kodak Co.) was recrystallized twice from water. Whenever possible other reagents were analytical grade. Glass-distilled water was used throughout.

*Buffers.* Phosphate buffers were prepared by neutralizing $KH_2PO_4$ with KOH; tris (Trizma Base, Sigma Chemical Co.) was neutralized with HCl.

## METHODS

*Homogenizing and centrifuging.* All homogenizing was done by hand, in glass homogenizers with tight-fitting Teflon pestles. Centrifuging, except where otherwise indicated, was carried out in an MSE Minor bench centrifuge at room temperature.

*Enzyme assays.* The enzymes mentioned below were assayed in a Beckman DK-2 recording spectrophotometer at 340 m$\mu$, temperature 25°. Unless otherwise stated, the assay systems (final vol. 2 ml.) contained components as listed below.

Citrate synthase: tris, pH 7·8, 50 mM; L-malate, 25 mM; NAD, 0·25 mM; acetyl-CoA, 0·09 mM; malate dehydrogenase, 50 $\mu$g.; synthase, 0·5–3 $\mu$g. The reaction was started with either acetyl-CoA or synthase.

Citrate ATP lyase: tris, pH 7·4, 50 mM; 2-mercaptoethanol, 10 mM; $MgCl_2$, 10 mM; ATP, 5 mM; CoA, 0·1 mM; NADH, 0·15 mM; malate dehydrogenase, 50 $\mu$g.; enzyme, (0·5–2 mg. of protein); citrate (last addition), 10 mM.

'Fatty acid synthase': phosphate, pH 6·6, 50 mM; 2-mercaptoethanol, 2·5 mM; acetyl-CoA, 0·09 mM; NADPH, 0·2 mM; enzyme (approx. 1 mg. of protein); malonyl-CoA (last addition), 0·06 mM.

*Determination of coenzyme A.* A modification of the method of Wakil & Hübscher (1960) was used. To the neutralized sample to be assayed (0·3–0·6 ml. containing 2–30 $\mu$m-moles of CoA and approx. 5 mM-mercaptoethanol) was added 0·3 ml. of a solution (stored at −15°) containing 15 mM-potassium sorbate, 15 mM-ATP, 30 mM-$MgCl_2$ and 250 mM-tris buffer, pH 8·5. After addition of 20–50 $\mu$l. of acyl-CoA synthetase, the mixture (final vol. 0·95 ml.) was incubated at room temperature (about 20°) for 20–30 min.; the extinction at 300 m$\mu$ (1 cm. light-path) was then measured in a Unicam SP. 500 spectrophotometer. The reference cells differed only in that water replaced acyl-CoA synthetase; the extinction due to the synthetase itself (0·03–0·10) was measured separately in the absence of CoA, and was subtracted from that observed in the experimental cells. CoA concentrations were calculated assuming a molar extinction coefficient for sorboyl-CoA of 19·3 $cm.^2/\mu$mole (Wakil & Hübscher, 1960). Control experiments showed that formation of other sorboyl thio esters (Michal & Bergmeyer, 1963) was not leading to high CoA estimates.

*Determination of acetyl-coenzyme A.* Neutralized tissue extract (3 ml., fraction C, see below) was mixed with 1 ml. of a solution containing: L-malate, 100 mM; NAD, 4 mM; tris, pH 7·8, 300 mM. To the mixture, in a spectrophotometer cell (light-path 4 cm.), was added excess of malate dehydrogenase (50 $\mu$g.). After 2–3 min. the constant extinction at 340 m$\mu$ was measured in a Unicam SP. 500 spectrophotometer, and approx. 25 $\mu$g. of citrate synthase was added. Acetyl-CoA concentration was calculated from the net increase in extinction (0·03–0·12), after allowance for that due to synthase itself (about 0·01).

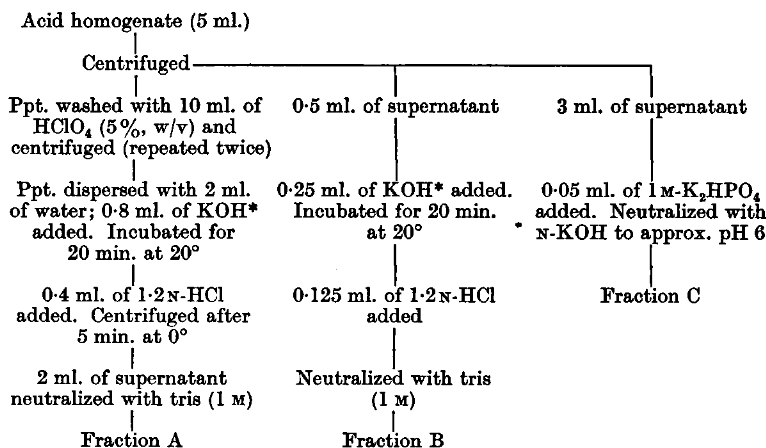
*Determination of acyl-coenzyme A.* Acyl-CoA compounds saturated in the 2,3-position (i.e. R·CH₂·CH₂·CO·S·CoA) were assayed with acyl-CoA dehydrogenase, by a method similar to that used by Hauge (1956). The assay system (final vol. 3 ml.) contained the following components: phosphate buffer, pH 7·0, 33 mM; cytochrome *c*, 50 $\mu$M; phenazine methosulphate, 6 $\mu$M; sample to be assayed (0–12 $\mu$m-moles of acyl-CoA); acyl-CoA dehydrogenase (about 0·75 mg. of protein). The enzyme was added to start the reaction, which was followed at 366 m$\mu$ (light-path 1 cm.; temp. 22–24°) in an Eppendorf photometer. A blank without acyl-CoA was also run, and the reaction was followed until the difference in extinction between experimental and control cells was constant. $\Delta E_{366\,m\mu}$ (oxidized – reduced) for cytochrome *c* is 14·4 cm.$^2$/$\mu$mole (Margoliash & Frohwirt, 1959); since acyl-CoA reduces 2 mol.prop. of cytochrome *c* the sensitivity is very high. Palmitoyl-carnitine was found not to react in this assay.

*Concentrations of coenzyme A and derivatives in rat tissues.* Rats were killed by decapitation, and liver samples immediately removed and placed in acetone containing solid $CO_2$. The frozen tissue was powdered in a percussion mortar pre-cooled with solid $CO_2$. Portions of the powder were rapidly mixed with 9 ml. (9·2 g.) of ice-cold 5% (w/v) $HClO_4$, and the weight of the powder (0·8–2·5 g.) was determined. The tissue was then homogenized in the acid, and samples of homogenate were processed as shown in Scheme 1: fraction C was centrifuged at 100000*g* for 45 min. to remove turbidity in tissue from non-starved rats.

The acid-insoluble CoA (assayed as CoA·SH in fraction A) was assumed to be combined with long-chain fatty acids and the CoA found in fraction B to be originally free CoA·SH or soluble derivatives, as discussed below. Concentrations of CoA in the original tissue were calculated by taking account of the dilutions involved in the procedures of Scheme 1, and were expressed as $\mu$m-moles/g. of frozen liver. Control experiments, in which whole (non-centrifuged) homogenate was processed by the method outlined for fraction B, were frequently done, and the results thus obtained for the total liver CoA agreed (within ±10%) with the total derived by adding the results obtained from fractions A and B. The figures for total CoA given in the Results section were obtained by adding the acid-soluble and -insoluble results.

Tests showed that very little CoA was destroyed during the procedures of Scheme 1. In view of the uncertainties in the extinction for sorboyl-CoA (Wakil & Hübscher, 1960, gave 19·3 cm.$^2$/$\mu$mole at 300 m$\mu$, and Michal & Bergmeyer, 1963, reported 23·5 cm.$^2$/$\mu$mole for *N*-acetyl-*S*-sorboylcysteamine), the absolute concentrations of CoA given may be in error by up to 20%; however, it is to relative, rather than absolute, values that attention has been mainly directed.

*Extraction of long-chain acyl-coenzyme A from liver.* Frozen and powdered rat liver was treated with $HClO_4$ (see fraction A, Scheme 1). The acid-washed precipitate was homogenized for 2 min. at 40–45° with 30 ml. of 0·2M-phosphate buffer, pH 6·0, containing bovine serum albumin (10 mg./ml.), and the residue was recovered by centrifuging at 13000*g* for 10 min. at 4°. This extraction procedure was repeated three times in all. The pooled supernatants were acidified with 3 ml. of $HClO_4$ (60%, w/v) and centrifuged. The precipitate was washed with 20 ml. of acetone, and extracted three times by homogenization for 2 min. at 45° with propan-2-ol–pyridine–water (1:1:1, by vol.), 40 ml. being used each time. The combined extract was rapidly (in less than 20 min.) dried in a rotary evaporator under $N_2$ at reduced pressure. The dry residue was suspended in 40 ml. of 40 mM-phosphate buffer, pH 6·0, kept at 0° for 5 min. and centrifuged. The supernatant was acidified with 2 ml. of 12N-HCl and centrifuged for 10 min. at 13000*g*. The small precipitate was washed with 20 ml. of acetone and collected by a similar centrifuging; this procedure was then repeated with ether. After drying in a stream of $N_2$ the residue was finally suspended in 3 ml. of 20 mM-phosphate buffer, pH 6·0. The CoA content of this fraction was determined as for fraction B (Scheme 1); in addition, acyl-CoA saturated in the 2,3-position was assayed as described above.




Scheme 1. Fractionation of acid-treated liver. Centrifuging was for 5 min. Solutions of KOH marked * were 0·85N, and contained 20mM-2-mercaptoethanol. Except where shown, the temperature was kept at 0°.

## RESULTS

*Concentrations of coenzyme A and derivatives in tissues of the rat.* (*a*) Liver. Table 1 shows the contents of total CoA, long-chain acyl-CoA and acetyl-CoA in the livers of control and starved rats, and starved rats fed with fat (beef suet) or carbohydrate (glucose or sucrose) for 24–48 hr. Contents of CoA in the livers of alloxan-diabetic rats are also shown. Results are expressed in both absolute terms and as a percentage of the total CoA; the latter method is independent of the large changes in liver weight which occur on starvation and refeeding. The 'acid-soluble CoA' includes free CoA·SH, soluble acyl derivatives (acetyl-CoA, succinyl-CoA, etc.), and also perhaps disulphides such as the mixed disulphide of CoA and glutathione. Preliminary experiments, in which this fraction was treated with *N*-ethylmaleimide (1 mM) at pH 6 and 20°, indicated that about half of the acid-soluble CoA was in fact CoA·SH; controls showed that acetyl-CoA was completely recovered after this treatment, and added CoA·SH was 80–100 % removed by alkylation. Thus a large proportion of the acid-soluble CoA is neither CoA·SH nor acetyl-CoA; its composition is being investigated.

CoA added to the acid homogenate of frozen tissue as the palmitoyl thio ester was quantitatively recovered as CoA·SH in fraction A of Scheme 1. In experiments designed to investigate the origin of the CoA in this fraction, it was found that over 90 % of the acid-insoluble CoA could be extracted from the tissue residue by the use of serum albumin solution, and partially purified as described in the Methods section. Thus the acid-insoluble CoA of liver resembled authentic palmitoyl-CoA with respect to liberation by alkali, binding by albumin, insolubility in acid, acetone and ether, and solubility in aqueous solution at pH 6·0. More direct evidence that the acid-insoluble CoA was long-chain acyl-CoA was obtained by assay with acyl-CoA dehydrogenase (Table 2). The differences between fed, starved and alloxan-diabetic rats were still evident in this direct assay.

(*b*) Heart. The assay of acid-soluble and -insoluble CoA has been extended without modification to the isolated perfused rat heart (Garland, Randle & Newsholme, 1963). Although the acid-

Table 1. *Coenzyme A in the livers of rats fed on a balanced diet, of similar rats after dietary alteration and after injection of alloxan*

Coenzyme A and its derivatives were assayed as described in the text. Results are means±S.E./g. of wet tissue. Figures in parentheses are the numbers of rats in each group.

| Condition of rats | Total CoA (μm-moles/g.) | Long-chain acyl-CoA (μm-moles/g.) | Long-chain acyl-CoA (% of total CoA) | Acid-soluble CoA (μm-moles/g.) | Acetyl-CoA (μm-moles/g.) | Acetyl-CoA (% of total CoA) |
|---|---|---|---|---|---|---|
| Balanced diet | 434±68 (7) | 52·8±8·6 (7) | 12·7±0·9 (7) | 385±61 (7) | 17·2±3·0 (5) | 4·0±1·2 (5) |
| Starved for 48 hr. | 513±80 (8) | 110±28 (8) | 21·4±4·8 (8) | 391±70 (8) | 39·2±3·9 (5) | 7·5±1·1 (5) |
| Starved for 48 hr., fed fat for 48 hr. | 426±53 (5) | 135±23 (5) | 30·1±1·1 (5) | 298±36 (5) | 25·1–32·0* (3) | 6·3–9·2* (3) |
| Starved for 48 hr., fed sugar for 48 hr. | 310±11 (5) | 30·4±9·5 (5) | 9·6±2·0 (5) | 277±12 (5) | 14·2±1·8 (5) | 4·4±0·8 (5) |
| Alloxan-diabetic (blood glucose 800–1000 mg./100 ml.) | 320±49 (6) | 92±20 (6) | 28·6±1·9 (6) | 227±29 (6) | 23·7±3·0 (5) | 6·9±0·8 (5) |

* Observed range.

Table 2. *Long-chain acyl-coenzyme A of rat liver extracted with bovine serum albumin*

The acyl-CoA was extracted from the acid-insoluble tissue residue and partially purified and assayed as described in the text.

| Individual rat | Condition of rats | Acid-insoluble CoA (μm-moles/g. of wet liver): Total extracted | Acid-insoluble CoA (μm-moles/g. of wet liver): Assayed as $RCH_2 \cdot CH_2 \cdot CO \cdot S \cdot CoA$ |
|---|---|---|---|
| 1 | Normal | 36 | 32 |
| 2 | | 38 | 38 |
| 3 | Starved for 36 hr. | 118 | 70 |
| 4 | | 121 | 62 |
| 5 | Alloxan-diabetic | 128 | 80 |
| 6 | | 98 | 63 |

insoluble CoA of heart muscle has not been further investigated to determine its nature, the increases found under conditions of increased fatty acid supply (starvation, alloxan diabetes and perfusion with palmitate) are compatible with the assumption that it is predominantly long-chain acyl-CoA (P. B. Garland & P. J. Randle, unpublished work).

*Effects of palmitoyl-coenzyme A on various enzymes.* (*a*) Fatty acid synthesis from malonyl-coenzyme A. The effect of increasing palmitoyl-CoA concentrations on the oxidation of NADPH is shown in Fig. 1. Under these conditions 50 % inhibition was caused by about 0·04 mM-palmitoyl-CoA; this was not relieved by increasing the acetyl-CoA concentration. Free palmitate (ammonium salt, 0·2 $\mu$mole/ml.) did not inhibit. Other experiments showed that the inhibition by palmitoyl-CoA was not competitive with respect to malonyl-CoA. Inhibition was prevented by bovine serum albumin (2 mg./ml.) when this was added before enzyme, but albumin was ineffective in reversing the inhibition (compare citrate synthase below).

(*b*) Citrate synthase. As reported by Tubbs (1963), and also by Wieland & Weiss (1963*b*), this enzyme is very sensitive to palmitoyl-CoA. Free CoA is very much less inhibitory, and palmitate (added as the ammonium salt) and palmitoyl-DL-carnitine cause no inhibition at 0·2 mM concentration. The inhibition is both prevented (Fig. 2) and reversed (Fig. 3) by serum albumin. From the data of Fig. 2 it may be calculated that 1 mole of serum albumin can tightly bind at least 15 moles of palmitoyl-CoA.

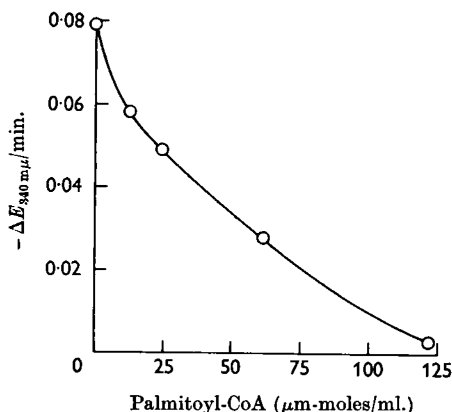



Fig. 1. Inhibition by palmitoyl-CoA of fatty acid synthesis from malonyl-CoA and acetyl-CoA. The rate of NADPH oxidation, determined in the system described in the Methods section, was taken as a measure of fatty acid synthesis. The assay system contained the indicated amounts of palmitoyl-CoA.

(*c*) Citrate adenosine triphosphate lyase. Kornacker & Lowenstein (1963) have reported a decreased activity of this enzyme in the livers of starved rats; refeeding, especially when a diet of bread is used (J. M. Lowenstein, personal communication), results in greatly increased activity. It has been found that refeeding with fat, or induction of alloxan diabetes, cause very low cleavage activity (P. K. Tubbs & P. B. Garland, unpublished work). Palmitoyl-CoA (but not acetyl- or butyryl-CoA) inhibits this enzyme, but the concentration required for 50 % inhibition is rather high (0·1 mM). Preincubation of the enzyme with 1 mM-palmitoyl-CoA does not increase the inhibition, which is reversible by dilution.

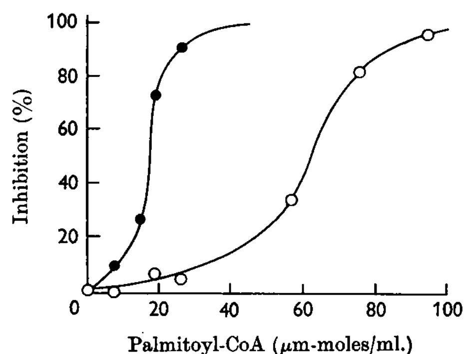



Fig. 2. Inhibition of citrate synthase by palmitoyl-CoA. Citrate synthase (0·89 $\mu$g. of protein) was incubated for 10 min. at 20° in the assay system (less acetyl-CoA) with the indicated amounts of palmitoyl-CoA. The reaction, started with acetyl-CoA, was followed at 25°. ○, No serum albumin; ●, with serum albumin (0·2 mg./ml.).

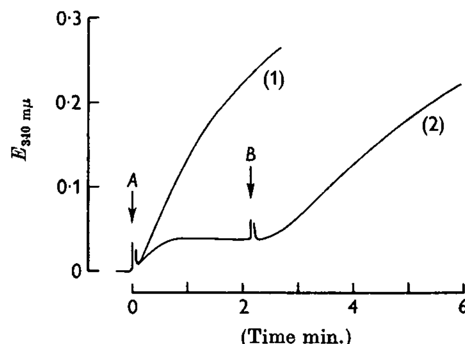



Fig. 3. Reversibility of the inhibition of citrate synthase by palmitoyl-CoA. The activity of synthase (3 $\mu$g. of protein, added at *A*) was measured as described in the Methods section. Curve (1), no palmitoyl-CoA; curve (2), with palmitoyl-CoA (30 $\mu$m-moles/ml.). Bovine serum albumin was added at *B* (final concn. 0·4 mg./ml.).

## DISCUSSION

It is well established that CoA plays a vital part in the oxidation and synthesis of fatty acids, and in their incorporation into lipids; it is also necessary for the oxidation of pyruvate and α-oxoglutarate, and is involved in the metabolism of several amino acids. In view of this, any shifts in the acylation state of CoA would be expected to have widespread effects on the pattern of cellular metabolism; conversely, observable changes in the steady-state concentrations of CoA derivatives should accompany metabolic disturbances. Until recently information on the behaviour of the coenzyme in tissues has been surprisingly lacking. Wieland, Löffler, Weiss & Neufeldt (1960) reported that β-hydroxy-β-methylglutaryl-CoA was elevated in the livers of starved and diabetic rats, and Wieland & Weiss (1963*a*) have found the same for acetyl-CoA. Besides confirming the latter observation, the present work provides evidence that large fluctuations occur in the contents of tissue long-chain fatty acid derivatives of CoA.

Since our preliminary communications (Tubbs & Garland, 1963; Garland & Tubbs, 1963), Bortz & Lynen (1963*b*) have reported an increase in the fatty acyl-CoA of liver after starvation; the magnitude of the change found was much the same as in the present work, but the contents were only a third as high. The difference may be due to the facts that these workers did not add a reducing agent (such as mercaptoethanol) during the alkaline hydrolysis of the acyl-CoA, and did not rapidly freeze the fresh tissue. The former omission may result in considerable loss of CoA by disulphide exchange with denatured protein (see Frater & Hird, 1963).

*Fatty acid synthesis.* Circumstances accompanied by reduced fatty acid synthesis (starvation, fat feeding, diabetes) are found to be associated with elevated fatty acyl-CoA contents in liver and heart. Refeeding starved animals with carbohydrate, known to result in abnormally high rates of fatty acid synthesis (Medes, Thomas & Weinhouse, 1952), caused a fall in liver fatty acyl-CoA concentrations to values below the controls (Table 1). The inhibition of 'fatty acid synthase' by acyl-CoA *in vitro* described above may be of physiological significance in the regulation of fatty acid synthesis, since the concentration of acyl-CoA required are comparable with the values found in tissues. The same applies to the competitive inhibition of acetyl-CoA carboxylase (EC 6.4.1.2) by long-chain acyl-CoA studied by Bortz & Lynen (1963*a*), especially since it is suggested that it is this step which is rate-limiting in fatty acid synthesis in the livers of normal and starved rats (Numa, Matsuhashi & Lynen, 1961), and which is impaired after fat feeding (Bortz, Abraham, & Chaikoff, 1963). Gibson & Hubbard (1960), on the other hand, found the 'fatty acid synthase' to be reduced in the livers of diabetic and starved rats. It may be that inhibition of both steps is of importance in different circumstances; no information is available as to the reversibility of the carboxylase inhibition, but the inhibition of 'fatty acid synthase' is not readily reversible *in vitro*. In any event, it appears probable that the concentration of long-chain acyl-CoA may in fact regulate the rate of fatty acid synthesis by processes of direct inhibition; the concentration of acyl-CoA may in turn be controlled by the relative availability of glycerol 1-phosphate and of fatty acids (Fritz, 1961; Masoro, 1962).

Since the microsomal fraction of rat liver contains both acyl-CoA synthetase and acyl-CoA hydrolase (Brandes, Olley & Shapiro, 1963), the varied effects of adding this fraction in experiments on fatty acid synthesis (e.g. Matthes, Abraham & Chaikoff, 1960; Masoro, Korchak & Porter, 1962) may be due to increases or decreases, according to the conditions used, in the concentrations of fatty acyl-CoA.

*Citrate metabolism and ketogenesis.* The significance of the inhibition of citrate synthase by acyl-CoA is not easy to assess. The details of citrate metabolism are especially important, since this compound, apart from being a tricarboxylic acid-cycle intermediate, directly affects at least two enzymes of regulatory interest, namely acetyl-CoA carboxylase (activation) (Martin & Vagelos, 1962) and phosphofructokinase (inhibition) (Passonneau & Lowry, 1963; Garland *et al.* 1963; Parmeggiani & Bowman, 1963). Furthermore, citrate itself has been proposed as an 'acetyl carrier', allowing intramitochondrial $C_2$ units to become available for extramitochondrial fatty acid synthesis (Srere & Bhaduri, 1962). In contradiction to the assumption made in an earlier communication (Tubbs, 1963), which was based on a report by Frohmann, Orten & Smith (1951), it appears that liver citrate concentrations are doubled or trebled by starvation (P. B. Garland, unpublished work) and diabetes (Parmeggiani & Bowman, 1963). In view of the fall in activity of liver citrate ATP lyase in starvation, alloxan diabetes and fat feeding, and its inhibition *in vitro* by palmitoyl-CoA, it would seem reasonable that at least part of the rise in liver citrate in these conditions may be caused by reduced citrate cleavage.

There is evidence that the flow through the tricarboxylic acid cycle is not impaired in conditions favouring ketone-body synthesis (Krebs, 1960); indeed the question arises as to why it does not increase. Several factors may contribute to the failure of citrate synthase to prevent an increase in

the liver acetyl-CoA concentration in these conditions: (i) Srere & Kosicki (1961*b*) have reported that at low oxaloacetate concentrations the activity of citrate synthase does not increase with elevated acetyl-CoA; (ii) accumulation of long-chain acyl-CoA may, by partial inhibition, prevent an increase in synthase activity; (iii) if the steady-state concentrations of the reactants of the synthase step (acetyl-CoA, oxaloacetate, CoA, citrate) are near their mass-action equilibrium concentrations elevated citrate will cause increased acetyl-CoA; (iv) under some conditions the oxaloacetate concentration may fall (Wieland & Löffler, 1963). Because of the nature of the initial steps in ketogenesis (condensation of two molecules of acetyl-CoA, giving acetoacetyl-CoA, followed by reaction with a third to give $\beta$-hydroxy-$\beta$-methylglutaryl-CoA; Lynen, Henning, Bublitz, Sörbo & Kröplin-Rueff, 1958), the observed increase in acetyl-CoA could result in a proportionally much greater increase in the rate of ketone-body synthesis.

## SUMMARY

1. Procedures are described for the estimation of total CoA, long-chain fatty acyl-CoA and acetyl-CoA in tissues. A method for determining the amount of CoA derivatives of fatty acids saturated in the 2,3-position is also given, and has been applied to tissue extracts.

2. The proportions of the total CoA of liver which are combined with long-chain fatty acids and with acetate are increased in rats which have been starved, starved and then fed with fat, or in which diabetes has been induced by alloxan.

3. Refeeding starved rats with sugar causes a decrease in the liver fatty acyl-CoA; the effect on acetyl-CoA is less marked.

4. The inhibition by palmitoyl-CoA of 'fatty acid synthase' and citrate ATP lyase (citrate cleavage enzyme) has been studied.

5. Citrate synthase (EC 4.1.3.7, condensing enzyme) is very strongly inhibited by palmitoyl-CoA. This inhibition may be prevented or reversed by serum albumin.

6. The effects on cellular metabolism of variations in the acylation state of CoA are discussed. In particular, it appears likely that the rate of fatty acid synthesis may be directly regulated by the concentration of tissue long-chain acyl-CoA. The effect of alteration in acetyl-CoA concentration on ketogenesis is mentioned, and the relationship in liver between acetyl-CoA and citrate is discussed.

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# Purification and Properties of Acyltransferases from *Pseudomonas aeruginosa*

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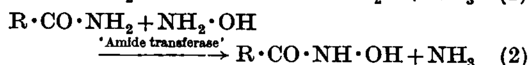
*Pseudomonas aeruginosa*, unlike other pseudomonads, possesses the ability to hydrolyse acetamide (Bühlmann, Vischer & Bruhin, 1961) and to utilize a variety of aliphatic amides as sole carbon and nitrogen source for growth; both these properties are associated with the presence of inducible amidases (Kelly & Clarke, 1962). The rate of appearance of amidase activity in succinate-grown *P. aeruginosa* 8602/A adapting to growth on acetamide has been shown to be discontinuous and to proceed in two distinct and separable phases (Kelly & Kornberg, 1962*a*). In phase I, amidase was produced before the onset of growth on acetamide: this phase was completed when, as a consequence of amidase action, the acetamide content of the medium fell to a low level. In phase II, amidase was produced, rapidly but gratuitously (cf. Monod & Cohn, 1952), during the subsequent growth on the acetate that was formed through hydrolysis of acetamide. Adaptation of succinate-grown cells to growth on acetate was accompanied by phase II but not phase I of amidase formation: this production of amidase was abolished or arrested by the addition of 5 mM-cyanoacetamide to the medium. In contrast, cyanoacetamide at this concentration did not affect the formation of amidase necessary for growth on acetamide (phase I).

These observations suggested that the nature of the carbon source influenced either the nature of the amidase(s) formed or the mode of their formation. Since preliminary purification of the enzyme (Kelly & Kornberg, 1962*b*) produced by acetate-grown cells (phase II) had shown it to catalyse not only the hydrolysis of aliphatic amides (reaction 1) but also the transfer of the acyl moiety of such amides (reaction 2) or of the corresponding acids (reaction 3) to hydroxylamine, it was feasible to test the possible identity of the enzyme produced in phase I by comparing its ability to effect the following three reactions:

$$R\cdot CO\cdot NH_2 + H\cdot OH \xrightarrow{\text{'Amidase'}} R\cdot CO_2H + NH_3 \quad (1)$$



$$R\cdot CO\cdot NH_2 + NH_2\cdot OH \xrightarrow{\text{'Amide transferase'}} R\cdot CO\cdot NH\cdot OH + NH_3 \quad (2)$$

$$R\cdot CO_2H + NH_2\cdot OH \xrightarrow{\text{'Acid transferase'}} R\cdot CO\cdot NH\cdot OH + H_2O \quad (3)$$

The results obtained indicate that the enzyme produced in phase I is identical with that produced in phase II; since the amidase activity (reaction 1) is but one example of the ability to transfer the acyl moieties to suitable acceptors, it is suggested that these enzymes be designated 'aliphatic acyltransferases' rather than 'amidases'.

Similar findings have been obtained by W. B. Jakoby & Joan D. Fredericks (personal communication).

## MATERIALS AND METHODS

*Maintenance and growth of the organisms.* Cultures of *P. aeruginosa* 8602/A were maintained on slopes containing minimal salts medium (Kelly & Clarke, 1962) plus acetamide (50 mM), solidified with 2% (w/v) Ionagar no. 2 (Oxo Ltd., London). The organisms were subcultured at weekly intervals, grown at 37° and stored at 5°.

For growth in liquid media, loopfuls of bacteria from a freshly grown slope were inoculated into 2 l. conical flasks, each containing 1 l. of minimal salts medium with either a mixture of 80 mM-sodium acetate and 80 mM-ammonium

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