# Studies of Fatty Acid Oxidation

## 3. THE EFFECTS OF ACYL-CoA COMPLEXES ON FATTY ACID OXIDATION*

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The oxidation of fatty acids to acetoacetate or to carbon dioxide is affected by the presence of various aliphatic and aromatic acids. For example, benzoate inhibits acetoacetate formation from butyrate at low concentrations in guinea pig liver slices (Jowett & Quastel, 1935*b*) and in 'cyclophorase' preparations (Grafflin & Green, 1948). Benzoate also inhibits the oxidation of butyrate to $CO_2$ in guinea pig liver slices (Avigan & Scholefield, 1954) and the oxidation of alkylthio fatty acids to $CO_2$ in rat-liver slices (Brown & Scholefield, 1954). Again, propionate markedly inhibits the formation of acetoacetate from butyrate, in guinea pig liver slices, where it is rapidly oxidized (Quastel & Wheatley, 1933). Liver 'cyclophorase' preparations, when well washed, do not oxidize propionic or phenylpropionic acid (Grafflin & Green, 1948), but in such preparations the presence of propionate still produces an inhibition of acetoacetate synthesis from acetate (Lang & Bässler, 1953). It follows, therefore, that propionate exercises its inhibition by a mechanism other than by formation of pyruvate.

The alkylthio fatty acids also inhibit acetoacetate formation from acetate and butyrate in guinea pig liver slices, probably by first forming CoA derivatives (Avigan & Scholefield, 1954). Similarly, benzoate may inhibit acetoacetate synthesis as a result of benzoyl-CoA formation.

The purpose of the present paper is to throw light on the mechanism of inhibition of acetoacetate synthesis by acyl-CoA complexes and to offer an explanation of the effects of various carboxylic acids on fatty acid metabolism. An attempt will also be made to explain the fact that, in presence of sources of oxaloacetate, the long-chain fatty acids have a greater tendency than the short-chain fatty acids to be oxidized through the citric acid cycle (Kennedy & Lehninger, 1950).

## EXPERIMENTAL

*Materials.* Commercial samples of coenzyme A (Pabst) and lithium acetyl phosphate (Schwarz Laboratories) were used. The potassium salts of butyric, octanoic, palmitic and the ethylthio fatty acids were purified by recrystallization from ethanol. *Escherichia coli* extracts were made by the method of Hughes (1951) from organisms grown in a yeast extract–peptone broth. Mitochondria were prepared by the method of Schneider (1948) using 0·25M sucrose and incubated in the medium described by Judah & Rees (1953). Acetone powders of mitochondria were prepared by the method of Drysdale & Lardy (1953). Pigeon-liver acetone powders were obtained by standard procedures and stored in a vacuum desiccator at 0°.

*Analytical methods.* Determinations of sulphanilamide were made by the method of Bratton & Marshall (1939), and of acetoacetate by the method of Jowett & Quastel (1935*a*) modified, as previously described (Avigan & Scholefield, 1954) for estimation of radioactive acetoacetate.

## RESULTS

### *Effects of fatty acid-CoA complexes on acetoacetate synthesis*

Earlier results have indicated that sodium ethylthioacetate and sodium benzoate probably inhibit the synthesis of acetoacetate by competition of their coenzyme A (CoA) derivatives with acetyl-CoA (Avigan & Scholefield, 1954). The possibility that the coenzyme A derivatives of the short-chain fatty acids inhibit acetoacetate synthesis was, therefore, examined.

The system employed was similar to that used by Avigan & Scholefield (1954), except that extracts of acetone powders of guinea pig liver mitochondria were used for effecting acetoacetate synthesis. The inhibitions of acetoacetate synthesis were found to be largest in this system. Typical results obtained are given in Table 1.

All the carboxylic acids tested formed coenzyme A derivatives as determined by the hydroxamic acid test (Lipmann & Tuttle, 1945), acetate forming the least amount of hydroxamic acid and octanoate the most. Nevertheless, when the synthesis of [*carboxy*-$^{14}$C]acetoacetate was estimated, it was apparent that all the compounds tested produced an inhibition of the synthesis of acetoacetate and that the extents of inhibition bore no relationship to the relative amounts of acyl-CoA complexes formed. It has already been shown that alkylthioacetyl-CoA complexes inhibit the various reactions of acetyl-CoA to varying extents (Avigan & Scholefield,

* Paper 2 of this series: Avigan & Scholefield, 1954, 58, 374.

1954). The nature of the group attached to the CoA greatly influences the extent of inhibition.

Butyrate has little or no inhibitory effect on acetoacetate synthesis from acetate in presence of extracts of acetone powders of pigeon liver, indicating that it exercises no inhibitory effect on the enzyme responsible for acetyl-CoA condensation to acetoacetate. If, however, for the pigeon-liver preparation there is substituted an extract of acetone powder of guinea pig liver mitochondria, there is a large inhibition of acetoacetate synthesis by butyrate (Table 1). The significant difference between the two preparations is that the latter is able to synthesize butyryl-CoA, whereas the necessary enzyme system is absent from the pigeon-liver preparation. Typical results illustrating the inhibition of acetoacetate synthesis in the guinea pig liver preparation and the capacity of this preparation to bring about the synthesis of various CoA complexes are shown in Table 1.

Table 1. *Effects of carboxylic acids on acetoacetate formation from acetate*

Each Warburg manometric vessel contained 60 $\mu$moles aminotrishydroxymethylmethane (THAM) buffer, pH 8·0; 30 $\mu$moles cysteine pH 8·0; 15 $\mu$moles ATP and 1·0 ml. enzyme solution equivalent to 50 mg. acetone powder of guinea pig liver mitochondria in 0·018 M magnesium chloride solution.

For assay of acetoacetate formation, 15 $\mu$moles [*carboxy*-$^{14}$C]acetate (10 000 counts/min./$\mu$mole) and 15 $\mu$moles carboxylic acid were added as indicated below. For the assay of hydroxamic acid formation, 15 $\mu$moles carboxylic acid (but no radioactive acetate) and 600 $\mu$moles hydroxylamine, pH 8·0 were added to each vessel. Final volume in both assays 3 ml.; incubation time, 1 hr. at 37°. All neutralizations were made with KOH solution.

| Carboxylic acid added | [*carboxy*-$^{14}$C]-Acetoacetate formed ($\mu$moles) | Inhibition (%) | Hydroxamic acid* formed ($\mu$moles) |
|---|---|---|---|
| Acetic† | 0·056 | — | 0·43 |
| Propionic | 0·012 | 79 | 0·81 |
| Butyric | 0·009 | 84 | 1·12 |
| Octanoic | 0·011 | 80 | 2·08 |
| Ethylthioacetic | 0·023 | 59 | 1·01 |
| Benzoic | <0·001 | >98 | 0·80 |

* Estimated from a standard curve for acethydroxamic acid.

† No additional unlabelled acetate is added in the acetoacetate assay.

*Acetylation of sulphanilamide*

The effects of potassium butyrate (0·01 M) and potassium benzoate (0·01 M) on the acetylation of sulphanilamide are shown in Table 2. Pigeon-liver extract was the source of the complete sulphanilamide acetylation enzyme system, and an extract of an acetone powder of guinea pig liver mitochondria provided the system forming acyl-CoA. Sodium acetate and adenosine triphosphate (ATP) were added as a source of the acetyl group. Neither butyrate nor benzoate at the concentrations quoted had any effect on the rate of acetylation of sulphanilamide in pigeon-liver extract alone but potassium butyrate consistently inhibited sulphanilamide acetylation in the combined systems by 50 %. Potassium benzoate (0·01 M) effected, however, an inhibition of only 20 %. In contrast, the results given in Table 1 show that a greater inhibition of acetoacetate synthesis is effected by benzoate than by butyrate at equivalent concentrations (0·005 M).

Table 2. *The effects of butyrate and benzoate on the acetylation of sulphanilamide*

Each vessel contained 15 $\mu$moles phosphate buffer, pH 7·4; 15 $\mu$moles potassium acetate; 4·5 $\mu$moles ATP; 15 $\mu$moles cysteine, pH 7·4; 50 $\mu$g. coenzyme A; 80 $\mu$g. sulphanilamide and extracts containing the equivalent of 50 mg. acetone powder of pigeon liver and 25 mg. acetone powder of guinea pig liver mitochondria (both extracts treated with Dowex-1 resin). Total volume 1·5 ml., potassium-ion concentration constant in all vessels; incubation 1 hr. at 37°. Additions as indicated.

| Additions | Sulphanilamide acetylated ($\mu$g.) | |
|---|---|---|
| Nil | 27·5 | 45·5 |
| 15 $\mu$moles Potassium butyrate | 13·5 | — |
| 15 $\mu$moles Potassium benzoate | — | 35·5 |

*The effects of various concentrations of coenzyme A on acetylation reactions*

Kaplan & Lipmann (1948) have shown that the extent of sulphanilamide acetylation is dependent on the amount of CoA present in the system when this is present at low concentrations. Subsequent work has shown that excess CoA may actually inhibit reactions in which it is involved, for example, in acetoacetyl-CoA synthesis (Lynen & Ochoa, 1953) and in cholesterol synthesis (Migicovsky & Greenberg, 1954). In the present experiments, therefore, endogenous CoA was first removed from the tissue extracts by treatment with Dowex-1 resin (acid-treated form). A known quantity of CoA was then added to the systems.

Experiments show that increase in the amount of added CoA from 50 to 100 or 200 $\mu$g./vessel does not increase the rate of acetylation of sulphanilamide, nor does it reverse the inhibitory effects found on addition of the carboxylic acids. Simple removal of free coenzyme A, by formation of acyl-CoA complexes, with a subsequent decrease in the amount of acetyl-CoA synthesis, therefore, cannot explain these inhibitory effects. A further increase in the amount of coenzyme A to 500 $\mu$g./vessel produces a decrease

in the rate of sulphanilamide acetylation but the extent of inhibition is not large (Table 3). A similar amount, however, added to an extract of an acetone powder of guinea pig liver mitochondria, under the conditions given in Table 1, produced an inhibition of acetoacetate synthesis of 70% (Table 3). These results suggest that excess coenzyme A may inhibit some of the reactions of acetyl-CoA in a manner similar to that found for the acyl-CoA complexes.

Table 3. *The inhibition of acetylation by excess coenzyme A and its reversal*

For the measurement of sulphanilamide acetylation, each vessel contained 15 μmoles phosphate buffer, pH 7·4; 15 μmoles potassium acetate; 15 μmoles cysteine, pH 7·4; 15 μmoles lithium acetyl phosphate; 7·5 μmoles ATP; 80 μg. sulphanilamide and 0·5 ml. pigeon-liver extract in 0·018M magnesium chloride treated with Dowex-1 resin equivalent to 37·5 mg. acetone powder of pigeon liver. The extract of *Esch. coli* was thawed after crushing and 0·25 ml. used without dilution. Final volume 1·5 ml.; incubation time, 1 hr. at 37°. In the determination of acetoacetate synthesis, the conditions and concentrations were as in Table 1.

| Coenzyme A added (μg.) | Sulphanilamide acetylated: In absence of *Esch. coli* extract | Sulphanilamide acetylated: In presence of *Esch. coli* extract | Acetoacetate formed (counts/min. in carboxyl group) |
|---|---|---|---|
| 50 | 53 | 66 | 588 |
| 500 | 42 (20)* | 67 (0) | 192 (67) |

* Figures in parentheses refer to percentage inhibition.

*The reversal of inhibitions produced by coenzyme A and its acyl complexes*

If CoA and its esters actually compete with acetyl-CoA, then addition of the latter should reverse the inhibitory actions of excess coenzyme A and acyl-coenzyme A complexes. This conclusion was tested by using an extract of *Esch. coli* containing an active phosphotransacetylase system (Stadtman, Novelli & Lipmann, 1951) as a source of acetyl-CoA. This system produces relatively large quantities of acetyl-CoA, assayed by the hydroxamic acid method.

The results show that in the presence of this extract the rate of sulphanilamide acetylation is increased and the small inhibitory effect of excess CoA is completely reversed. These results are shown in Tables 3 and 4.

Reversal of the inhibition of sulphanilamide acetylation by butyryl-CoA is also obtained. Addition of potassium butyrate to the combined extracts of pigeon liver and of guinea pig liver mitochondria inhibited sulphanilamide acetylation by 50% and acetyl phosphate had no effect on this inhibition. Addition of acetyl phosphate together with *Esch. coli* extract, however, reduced the inhibition to 20% (Table 4). *Esch. coli* extracts are capable of synthesizing acetyl-CoA from acetate, ATP and CoA, in the absence of acetyl phosphate, and may effect partial reversal of the inhibition of sulphanilamide acetylation due to butyrate or benzoate (Table 4).

Table 4. *The inhibition of the acetylation of sulphanilamide by butyrate and its reversal by acetyl phosphate and* Esch. coli *extract*

Each vessel contained 15 μmoles phosphate buffer, pH 7·4; 15 μmoles potassium acetate; 4·5 μmoles ATP; 15 μmoles cysteine hydrochloride, pH 7·4; 50 μg. coenzyme A; 80 μg. sulphanilamide and 0·5 ml. extract (treated with Dowex-1 resin) in 0·018M magnesium chloride. The extract was equivalent to 50 mg. acetone powder of pigeon liver and 25 mg. acetone powder of guinea pig liver mitochondria. Final volume 1·5 ml.; total potassium-ion concentration constant; incubation time, 1 hr. at 37°.

| Additions: Potassium butyrate (μmoles) | Additions: *Esch. coli* extract (ml.) | Additions: Lithium acetyl phosphate (μmoles) | Sulphanilamide acetylated (μg.) |
|---|---|---|---|
| 0 | 0 | 0 | 31·0 |
| 15 | 0 | 0 | 16·0 |
| 0 | 0·15 | 0 | 22·5 |
| 15 | 0·15 | 0 | 16·5 |
| 0 | 0 | 15 | 33·5 |
| 15 | 0 | 15 | 18·5 |
| 0 | 0·15 | 15 | 38·0 |
| 15 | 0·15 | 15 | 30·5 |

*Mitochondrial oxidation of fatty acids*

The possibility that the presence of acyl-CoA complexes influences the rate of condensation of acetyl-CoA with various acetyl acceptors (e.g. acetyl-CoA itself, sulphanilamide or oxaloacetate) clearly has a bearing on problems of fatty acid oxidation.

The long-chain fatty acids, such as palmitic acid, produce proportionately less acetoacetate, in certain mitochondrial preparations, than the short-chain fatty acids (Kennedy & Lehninger, 1950). This relative inability to form acetoacetate might be due to an inhibition of acetoacetate synthesis by the CoA derivatives of the long-chain fatty acids. The effects of potassium palmitate (0·0017M), octanoate (0·0017M) and ethylthioacetate (0·0033M) on oxidation of potassium [*carboxy*-$C^{14}$]butyrate (0·0017M) in rat-liver mitochondria were, therefore, studied. Typical results obtained are shown in Table 5.

They show that (1) the presence of potassium octanoate inhibits about equally (90%) both $CO_2$ formation and acetoacetate synthesis from butyrate, and (2) the presence of either potassium palmitate or potassium ethylthioacetate causes a

much larger inhibition of acetoacetate synthesis than of $CO_2$ formation from butyrate.

Potassium octanoate is rapidly oxidized by preparations of rat-liver mitochondria and it is unlikely, therefore, that a relatively high concentration of octanoyl-CoA will accumulate. Hence it is not to be expected that a specific inhibitory effect of octanoyl-CoA, under these conditions, will occur. However, octanoate on oxidation gives rise to relatively large amounts of acetyl-CoA, which dilute the carbonyl-labelled acetyl-CoA formed from the [*carboxy*-$^{14}$C]butyrate. The effect of this dilution is to decrease equally the production of $^{14}CO_2$ and [*carboxy*-$^{14}$C]acetoacetate. The results of the experiment quoted in Table 5 show that approximately 90% decrease in the radioactivity of both products takes place. Similarly, in an experiment with guinea pig liver slices using 0·01 M [*carboxy*-$^{14}$C]butyrate and 0·001 M octanoate, 55% inhibition of both $^{14}CO_2$ and [*carboxy*-$^{14}$C]acetoacetate was obtained.

*Comparison of effects of benzoate and ethylthioacetate on butyrate oxidation*

The oxidation of [*carboxy*-$^{14}$C]butyrate by rat-liver mitochondria in the presence of several concentrations of ethylthioacetate and benzoate was also examined (Fig. 1). With increase in the amount of inhibitor added there is a decrease in the production of labelled acetoacetate. Benzoate is more effective in this respect than ethylthioacetate, and even at very low concentrations it inhibits acetoacetate synthesis with little or no effect on $CO_2$ formation. This points to a much higher inhibitory effect of benzoyl-CoA than of ethylthioacetyl-CoA on acetoacetate formation from butyrate. A significant increase in the production of $^{14}CO_2$ from carboxyl-labelled fatty acids by rat-liver mitochondria is often obtained in presence of ethylthioacetic acid (see Fig. 1).

Table 5. *The influence of carboxylic acids on the oxidation of potassium* [carboxy-$^{14}$C]*butyrate by rat-liver mitochondria*

The incubations were made in Warburg manometric vessels in the medium described by Judah & Rees (1953). Mitochondria obtained from 8 g. rat liver were finally suspended in 10 ml. 0·25 M sucrose; 0·7 ml. suspension, 5 $\mu$moles potassium butyrate (specific activity 900 counts/min./$\mu$mole), and 2 $\mu$moles malate were added per vessel. Final volume 3 ml.

| Further additions | $\mu$l. Oxygen taken up in 30 min. at 37° | $^{14}CO_2$ as total counts/min. | Acetoacetate carboxyl $^{14}$C as total counts/min. |
|---|---|---|---|
| Nil | 119 | 666 | 1190 |
| 5 $\mu$moles Potassium octanoate | 239 | 53 (92)* | 131 (89) |
| 5 $\mu$moles Potassium palmitate | 183 | 506 (24) | 565 (53) |
| 10 $\mu$moles Potassium ethylthioacetate | 92 | 490 (26) | 406 (66) |

* Figures in parentheses refer to percentage inhibition.

Potassium ethylthioacetate is not oxidized by rat-liver mitochondria, but in its presence the metabolism of [*carboxy*-$^{14}$C]butyrate is affected. The effect is similar to that obtained with guinea pig liver slices (Avigan & Scholefield, 1954), where it is suggested that the selective inhibitory effect of ethylthioacetate on acetoacetate synthesis may only be obtained after formation of ethylthioacetyl-CoA.

Potassium palmitate gives results (Table 5) which are intermediate between those obtained with potassium octanoate and those obtained with potassium ethylthioacetate. An increase in the rate of oxygen uptake is secured, indicating oxidation of the added palmitate. There is definite inhibition of $^{14}CO_2$ production (24%) but a much greater inhibition of acetoacetate formation (53%) from labelled butyrate.

## DISCUSSION

It is apparent that inhibitions of the reactions of acetyl-CoA by alkylthioacyl-CoA and benzoyl-CoA are also shown by the CoA complexes of some of the short-chain fatty acids. Previous results have shown that ethylthioacetyl-CoA inhibits the synthesis of free acetoacetate resulting from the condensation of acetyl-CoA with another molecule of acetyl-CoA, slightly inhibits the condensation of acetyl-CoA with sulphanilamide, and has little effect on its condensation with oxaloacetate as measured by metabolic $CO_2$ production. It has to be borne in mind, however, that CoA complexes may affect the hydrolysis of acetoacetyl-CoA and in this manner inhibit the liberation of free acetoacetate.

Benzoyl-CoA has a greater inhibitory effect than ethylthioacetyl-CoA on acetoacetate synthesis, sulphanilamide acetylation and metabolic $CO_2$

production at equivalent concentrations. Butyryl-CoA inhibits both sulphanilamide acetylation and acetoacetate production. Excess coenzyme A, itself, also inhibits acetoacetate synthesis and has a small effect on sulphanilamide acetylation. The magnitude of these inhibitions, therefore, depends on the nature of the CoA complex.

With acetoacetate synthesis the inhibitory effect of excess CoA is probably due mainly to a mass action effect on the equilibrium reaction catalysed by thiolase (Lynen & Ochoa, 1953):

$$CH_3.CO.CH_2.CO.S.CoA + CoA.SH \rightleftarrows 2CH_3.CO.S.CoA.$$

The possibility of a direct competition between CoA and its acetyl derivative, however, has not been ruled out, and the mass action effect does not account for the inhibitions produced by the CoA seems to eliminate the possibility that the inhibitions are due to a decrease in the concentration of acetyl-CoA, resulting from a simple exchange reaction, such as

$$R.COOH + CH_3CO.SCoA = RCO.SCoA + CH_3.COOH$$

as suggested for a bacterial system by Kennedy & Barker (1951). The exchange reactions postulated by Beinert & Stansly (1953) and by Lynen & Ochoa (1953), to account for asymmetric labelling of acetoacetate, cannot account for the fact that an absolute decrease in the rate of acetoacetate synthesis takes place on addition of benzoate to guinea pig liver slices (Jowett & Quastel, 1935*a*, *b*) or on addition of alkylthio acids to rat-liver slices or mitochondria (Scholefield, unpublished), in the presence of salts of fatty acids.

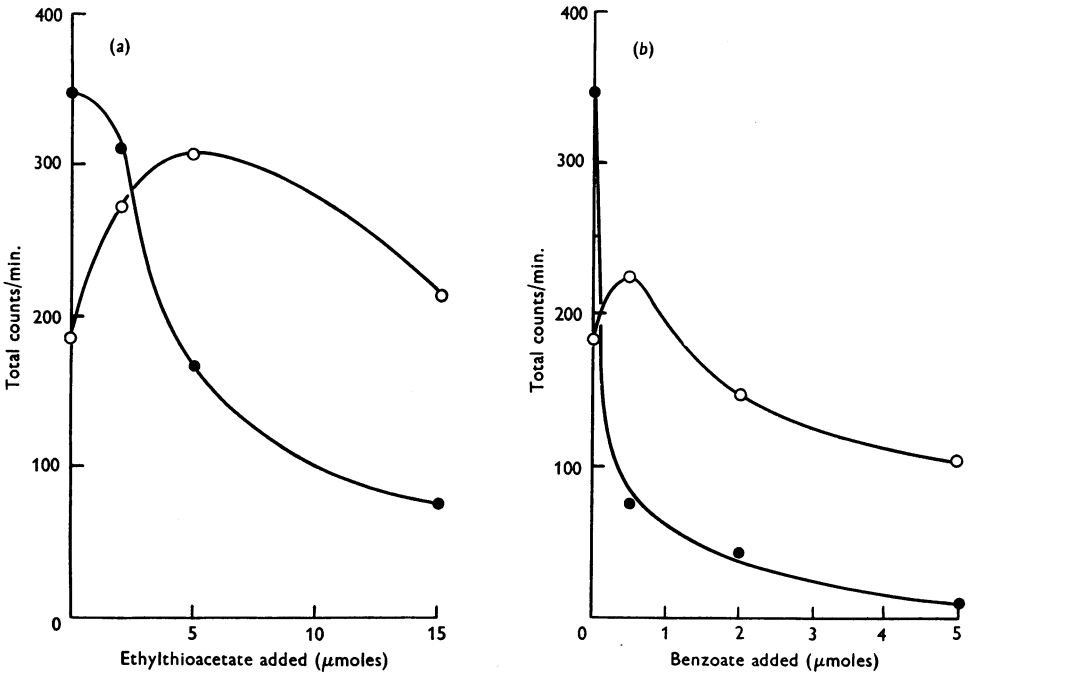


Fig. 1. The effects of various concentrations of ethylthioacetate and benzoate on the oxidation of [*carboxy*-$^{14}$C]butyrate by rat-liver mitochondria to $^{14}CO_2$ and [*carboxy*-$^{14}$C]acetoacetate. Conditions as described in Table 5. ○, Metabolic $^{14}CO_2$; ●, [*carboxy*-$^{14}$C]acetoacetate, as measured by decarboxylation to $^{14}CO_2$.

complexes of the carboxylic acids. In either case, excess acetyl-CoA should reverse such inhibitions. Reversal of the inhibition of sulphanilamide acetylation has been obtained by producing excess acetyl-CoA *in situ* with the phosphotransacetylase system of *Esch. coli* extract.

The fact that the relative inhibitory effects of acyl-CoA complexes on acetylation reactions depend upon the nature of the acetylation reaction

The CoA derivatives of the long-chain fatty acids may accumulate sufficiently to inhibit acetoacetate formation, thus accounting for the results obtained with palmitate in tissue slices and mitochondria. The presence of the long-chain fatty acids, therefore, should affect the oxidation of short-chain fatty acids, producing a greater effect on acetoacetate synthesis than on carbon dioxide production. The results obtained are in accord with this prediction.

They cannot be explained as being solely due to a dilution effect, since addition of octanoate results in an equal decrease in the formation of both products, whereas addition of palmitate results in an inhibition of acetoacetate synthesis with relatively little effect on $^{14}CO_2$ production. The results obtained with ethylthioacetate are similar to those obtained with palmitate but ethylthioacetate does not form acetyl-CoA, so that a dilution effect with this substance may be eliminated. Further, it has been shown by Tabor, Mehler & Stadtman (1953) that synthetic palmityl-CoA and CoA itself both inhibit the acetylation of *p*-nitroaniline by synthetic acetyl-CoA. It seems likely, therefore, that the presence of palmityl-CoA affects the rate of formation of acetoacetate in animal tissues.

## SUMMARY

1. The addition of fatty acids, such as butyric and octanoic, produces an inhibition of acetoacetate synthesis in extracts which are capable of forming acyl-CoA derivatives from fatty acids. Similar effects on sulphanilamide acetylation may also be obtained.

2. The effects of acyl-CoA compounds on the acetylation of sulphanilamide are reversed by acetyl-CoA prepared *in situ* from acetyl phosphate in the presence of *Esch. coli* extracts.

3. Studies on the oxidation of carboxyl-labelled butyrate by respiring rat-liver mitochondria have confirmed the fact that other fatty acids may compete for the butyrate activating enzyme system and that, on oxidation of these fatty acids, isotopic dilution occurs.

4. The addition of compounds such as benzoate, ethylthioacetate and palmitate to rat-liver mitochondria in the presence of [*carboxy*-$^{14}$C]butyrate results in a considerable decrease in the amount of radioactive acetoacetate formed but in little effect on the amount of radioactive $CO_2$ produced.

5. It is suggested that these results are consistent with the hypothesis that various reactions of acetyl-CoA (and possibly of acetoacetyl-CoA) are specifically inhibited by acyl-CoA compounds and that these inhibitory effects influence the kinetics of fatty acid oxidation to acetoacetate and carbon dioxide.

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# Hypoglycin A and B, two Biologically Active Polypeptides from *Blighia sapida*

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Several studies involving qualitative tests on animals (Bowrey, 1887; Connal & Ralston, 1918; Lynch, Larson & Doughty, 1951; Evans & Arnold, 1938; Jordan & Burrows, 1937) have indicated the presence of water-soluble toxic material in sections of the fruit of *Blighia sapida*, a common article of diet in Jamaica. Particular interest attaches to these observations in view of the suggestions by Scott (1916) that these fruit are responsible for 'vomiting sickness', an important local disease of undefined etiology (Hill, 1952; Jelliffe & Stuart, 1954). Although this view has not been generally accepted (Williams, 1952), it is supported by a considerable weight of circumstantial evidence and appeared worthy of closer examination.