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Studies on Medium-Chain Fatty Acyl-Coenzyme A Synthetase

ENZYME FRACTION II: MECHANISM OF REACTION AND SPECIFIC PROPERTIES

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1. The mechanism of reaction of fatty acyl-CoA synthesis catalysed by fatty acyl-CoA synthetase from ox liver (fraction II; Bar-Tana, Rose & Shapiro, 1968) was investigated by a kinetic study of CoA disappearance dependent on butyrate plus ATP or butyryl-AMP (overall and partial reaction *b* respectively). 2. Contrary to findings with another enzyme (fraction I), a Bi Uni Uni Bi Ping Pong mechanism (Cleland, 1963*a*,*b*,*c*) corresponding to Berg's (1956) scheme of reaction was eliminated and an ordered Ter Ter mechanism with an A–C–B (standing for ATP, CoA and butyrate respectively) sequence of substrate entry for the overall reaction was established for fraction II. Partial reaction (*b*) was found to follow the 'Iso-Theorell–Chance' mechanism. 3. Also, in contrast with results obtained with fraction I, no allosteric properties could be demonstrated with fraction II.

Fractionation of an extract from ox liver particle acetone-dried powder results in two separate enzyme fractions I and II (Bar-Tana, Rose & Shapiro, 1968) capable of catalysing the synthesis of acyl-CoA from medium-chain fatty acids and CoA in the presence of ATP, according to the overall stoicheiometry determined by Mahler, Wakil & Bock (1953):

$$R\cdot CO_2H + ATP + CoASH \rightleftharpoons R\cdot CO\cdot SCoA + PP_i + AMP$$

Studies on the mode of action of fraction I were described in the preceding paper (Bar-Tana & Rose, 1968). The overall reaction was found to proceed according to a mechanism similar to that proposed by Berg (1956) for yeast acetyl-CoA synthetase (Bi Uni Uni Bi Ping Pong according to Cleland, 1963*a*,*b*,*c*). Further, fraction I behaves as an allosteric protein in both the overall and the two partial reactions (*a*) and (*b*): (*a*) formation of ATP from acyl-AMP and PP_i; (*b*) acyl-AMP-dependent CoA disappearance:

(*a*) $R\cdot CO_2H + ATP \rightleftharpoons R\cdot CO\cdot AMP + PP_i$

(*b*) $R\cdot CO\cdot AMP + CoASH \rightleftharpoons R\cdot CO\cdot SCoA + AMP$

where ATP, CoA, AMP, PP_i and butyryl-CoA act as allosteric ligands. Fraction II, the subject of the present paper, differs from fraction I in two respects: (i) no ATP is formed from acyl-AMP and PP_i in the presence of this fraction despite the occurrence of the overall reaction as well as partial reaction (*b*); (ii) ATP was found to be a potent inhibitor of the partial reaction (*b*) in contrast with its effect as an allosteric activator of fraction I.

The complete absence of ATP formation but presence of overall reaction and partial reaction (*b*) points to a mechanism of enzyme action different from that proposed by Berg (1956). Alternative mechanisms for acyl-CoA synthetase, according to which fatty acyl-AMP is not a true intermediate in the overall reaction though it could serve as an acyl donor, have been suggested in the past (Boyer, Mills & Fromm, 1959). In this case, reaction (*b*) would not be a partial reaction but merely be analogous to the overall reaction. In view of these considerations it was decided to study the intrinsic mechanism of both the overall reaction and reaction (*b*) as catalysed by fraction II. This was carried out by kinetic studies based on theoretical propositions for multisubstrate and multiproduct enzyme systems (Cleland, 1963*a*,*b*,*c*). The fact that ATP strongly inhibited fraction II whereas it served as an allosteric activator of fraction I warranted further studies with respect to the possible allosteric nature of fraction II. Such information, together with data relevant to the order of entry of the substrates and exit of the products of the reaction, would serve to clarify the relationship between the allosteric site and the active site of the enzyme.

EXPERIMENTAL

Materials, source and purification of enzyme and substrates, and methods of assay were as described in the

preceding papers (Bar-Tana *et al.* 1968; Bar-Tana & Rose, 1968). Pantotheine was obtained by the reduction of pantothine (obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.) with $NaBH_4$. Methods of kinetic measurements and analyses of kinetic data were as described in the preceding paper (Bar-Tana & Rose, 1968).

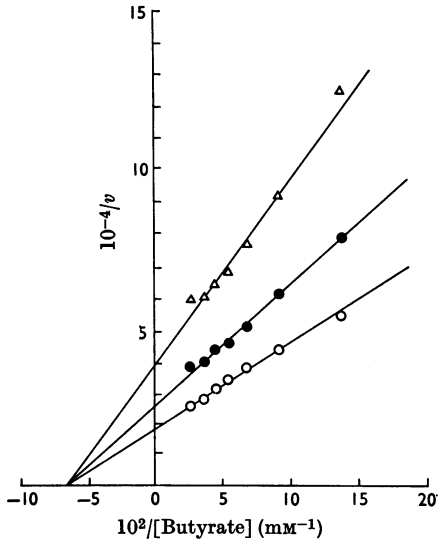


Fig. 1. Effect of CoA on double-reciprocal plots of various concentrations of butyrate, with ATP at saturating concentration. The incubation mixture contained: tris–HCl buffer, pH 8·0, 100mM; $MgCl_2$, 14mM; ATP, 12mM; CoA, as shown; sodium butyrate, as shown; enzyme (fraction II), 107μg. of protein/ml. of reaction mixture. △, 0·067mM-CoA; ●, 0·135mM-CoA; ○, 0·27mM-CoA. Incubation was for 10min. at 37°. v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

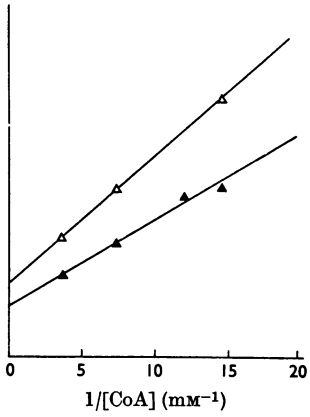


Fig. 2. Replot of slopes (△) and intercepts (▲) derived from Fig. 1 against the reciprocal of the concentration of CoA. The ordinate scales for (△) and (▲) are the ratio of ordinates to abscissa and the ordinate of Fig. 1 respectively.

RESULTS

Initial-velocity studies of the overall reaction. To establish or eliminate Berg's (1956) mechanism for the overall reaction, initial-velocity studies were undertaken. Measurements of reaction rates were carried out at variable concentrations of one of the substrates and different fixed concentrations of the

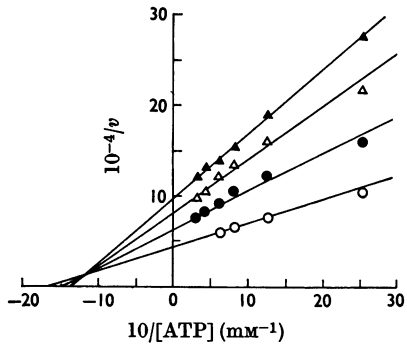


Fig. 3. Effect of CoA on double-reciprocal plots of various concentrations of ATP with butyrate at saturating concentration. The incubation mixture contained: tris–HCl buffer, pH 8·0, 80mM; $MgCl_2$, 11mM; sodium butyrate, 78mM; CoA, as shown; ATP, as shown; enzyme (fraction II), 88μg. of protein/ml. of reaction mixture. ▲, 0·047mM-CoA; △, 0·062mM-CoA; ●, 0·135mM-CoA; ○, 0·187mM-CoA Incubation was for 10min. at 37°. v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

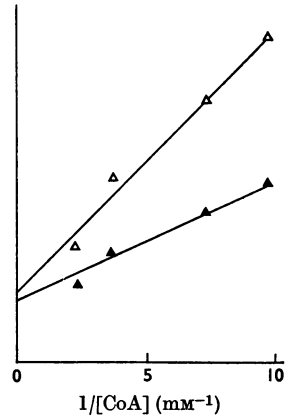


Fig. 4. Replot of slopes (△) and intercepts (▲) derived from Fig. 3 against reciprocal concentrations of CoA. Ordinate scales for (△) and (▲) are the ratio of ordinates to abscissa and the ordinate of Fig. 3 respectively.

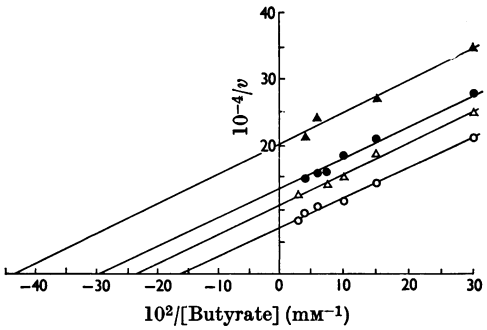


Fig. 5. Effect of ATP on double-reciprocal plots of various concentrations of butyrate with CoA at saturating concentration. The incubation mixture contained: tris–HCl buffer, pH 8·0, 80mM; $MgCl_2$, 16mM; CoA, 0·15mM; ATP, as shown; butyrate, as shown; enzyme (fraction II), 186μg. of protein/ml. of reaction mixture. ▲, 0·5mM-ATP; ●, 1mM-ATP; △, 1·33mM-ATP; ○, 2·5,M-ATP. Incubation was for 10min. at 37°. v=m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

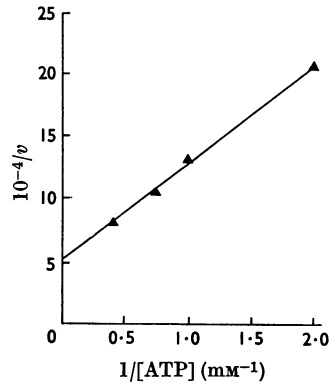


Fig. 6. Replot of intercepts derived from Fig. 5 against the reciprocal of the concentration of ATP.

second, that of the third substrate being kept constant. Where sodium butyrate was the variable substrate with ATP constant at saturating concentration and with several fixed concentrations of CoA, the double-reciprocal plots were linear, constituting a family of lines intersecting to the left of the vertical axis (Fig. 1). Replots of the slopes and intercepts against reciprocal concentrations of CoA were likewise linear (Fig. 2). The Michaelis constant for CoA was $1·7 \times 10^{-4}$M. When ATP was used as the variable substrate, at several fixed concentrations of CoA, and at saturating concentration of butyrate, the double-reciprocal plots were also linear and constituted a family of non-parallel lines, intersecting to the left of the vertical axis (Fig. 3). Replots of slopes and intercepts derived from these lines were also linear, demonstrating the absence of allosteric behaviour of fraction II towards CoA (Fig. 4). Again, when butyrate was variable and CoA kept at saturating concentration with several fixed concentrations of ATP, the double-reciprocal plots were linear, but this time constituted a family of parallel lines (Fig. 5). A replot of the intercepts against the reciprocal concentrations of ATP was likewise linear (Fig. 6). The Michaelis constants for ATP and butyrate derived from these results were $1·5 \times 10^{-3}$M and $1·0 \times 10^{-2}$M respectively.

Since the kinetic data obtained from the initial-velocity studies (Figs. 1–6) do not fit the Bi Uni Uni Bi Ping Pong mechanism, fraction II does not behave according to Berg's (1956) scheme of reaction, differing in this respect from fraction I (Bar-Tana & Rose, 1968). However, the data could be related to eqn. (1), describing the initial-velocity-rate law for a sequential Ter Ter mechanism, in which all three substrates must enter the reaction before any product is released (Cleland, 1963*a*,*b*,*c*).

$$v = \frac{VABC}{K_{ia}K_{ib}K_c + K_{ib}K_cA + K_{ic}K_bC + K_cAB + K_bAC + K_aBC + ABC} \qquad (1)$$

Assuming that A, B and C stand for the concentrations of ATP, butyrate and CoA respectively and other meanings are as defined by Bar-Tana & Rose (1968), the set of equations corresponding to the experimental conditions in Figs. 1, 3 and 5 are:

$$\frac{1}{v} = \frac{K_b}{V}\left(1+\frac{K_{ic}}{C}\right)\frac{1}{B} + \frac{1}{V}\left(1+\frac{K_c}{C}\right) \qquad (1a)$$

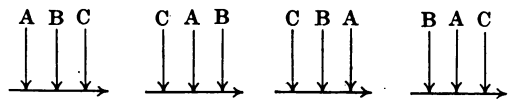
$$\frac{1}{v} = \frac{K_a}{V}\left(1+\frac{K_c}{C}\right)\frac{1}{A} + \frac{1}{V}\left(1+\frac{K_c}{C}\right) \qquad (1b)$$

$$\frac{1}{v} = \frac{K_b}{V}\cdot\frac{1}{B} + \frac{1}{V}\left(1+\frac{K_a}{A}\right) \qquad (1c)$$

These fit the kinetic data obtained. The pattern of linearity obtained in the double-reciprocal plots and replots of slopes and intercepts also eliminates the possibility of a sequential random mechanism in which alternative reaction sequences exist.

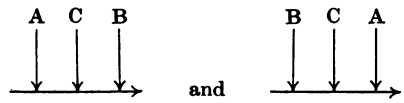
Out of six ways in which the three substrates A, B and C can enter the reaction in an obligatory

ordered fashion, the following four can be excluded because of the experimental data and its relationship to eqn. (1):



Thus the A–B–C and C–B–A sequences are excluded by the intersecting pattern obtained with butyrate at saturating concentrations (Fig. 3), and the B–A–C and C–A–B sequences are excluded by the intersecting pattern obtained at saturating concentrations of ATP (Fig. 1). Only with CoA at saturating concentration is a parallel pattern obtained (Fig. 5), showing that CoA enters between either ATP and butyrate or butyrate and ATP (Cleland, 1963*a*).

These two remaining possibilities:



cannot be distinguished by initial-velocity studies and analyses of product inhibition; binding studies or use of substrate analogues are required.

Initial-velocity and product-inhibition pattern of partial reaction (*b*). By the elimination of the possibility of the Ping Pong mechanism for the overall reaction, and in view of the sequential ordered mechanism suggested, the role of butyryl-AMP as an intermediate in the overall reaction, and hence the existence of reaction step (*b*) as a part of the overall reaction, became doubtful. Initial-velocity and product-inhibition studies of reaction (*b*) were therefore undertaken to clarify the mechanism involved. When CoA was used as the variable substrate, at several fixed concentrations of butyryl-AMP, the double-reciprocal plots were linear, and the family of straight lines obtained intersected to the left of the vertical axis (Fig. 7). The replots of the slopes and intercepts were also linear (Fig. 8), again demonstrating absence of allosteric behaviour of fraction II towards CoA. Interchanging the substrates, so that butyryl-AMP became the variable and CoA the fixed substrate, resulted in the same linear intersection pattern. The Michaelis constants for CoA and butyryl-AMP derived from these results were $1{\cdot}7 \times 10^{-4}$M and $8{\cdot}6 \times 10^{-4}$M respectively. The results obtained here fit eqn. (2), which is obeyed by several sequential Bi Bi mechanisms such as ordered Bi Bi, rapid-equilibrium random Bi Bi or Theorell–Chance (Cleland, 1963*a*,*b*,*c*):

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \qquad (2)$$

where V is the maximum velocity of reaction (*b*) in the forward direction, K_a and K_b are the respective Michaelis constants for A and B, and K_{ia} and K_{ib} are the inhibition constants for A and B.

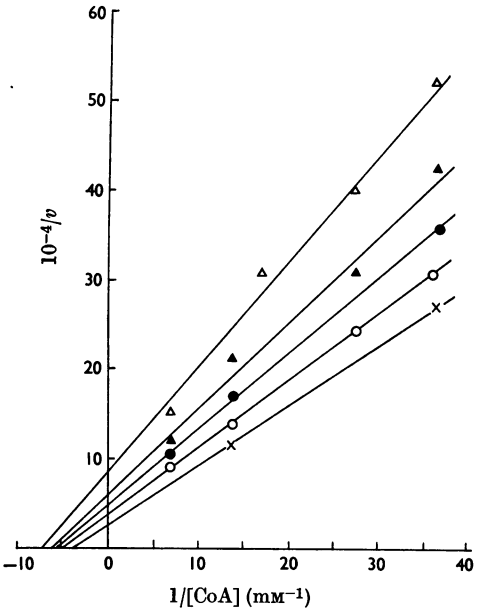


Fig. 7. Effect of butyryl-AMP on double-reciprocal plots of various concentrations of CoA. The incubation mixture contained: tris–HCl buffer, pH 8·0, 70mM; $MgCl_2$, 6mM; CoA, as shown; butyryl-AMP, as shown; enzyme (fraction II), 13·2μg. of protein/ml. of reaction mixture. △, 0·286mM-butyryl-AMP; ▲, 0·430mM-butyryl-AMP; ●, 0·645mM-butyryl-AMP; ○, 0·860mM-butyryl-AMP; ×, 1·29mM-butyryl-AMP. Incubation was for 10min. at 37°. v= m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

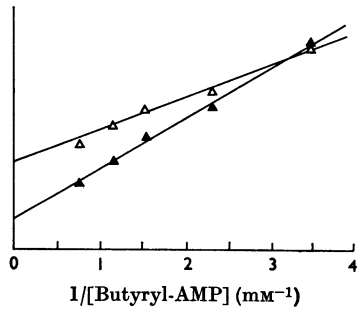


Fig. 8. Replot of slopes (△) and intercepts (▲) derived from Fig. 7 against reciprocal concentrations of butyryl-AMP. Ordinate scales for (△) and (▲) are the ratio of ordinates to abscissa and the ordinate of Fig. 7 respectively.

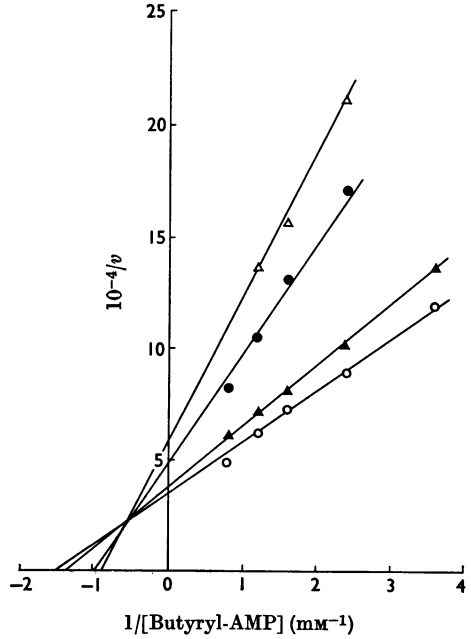


Fig. 9. Effect of AMP on double-reciprocal plot of various concentrations of butyryl-AMP with CoA constant at saturating concentration. The incubation mixture contained: tris–HCl buffer, pH 8·0, 70 mM; $MgCl_2$, 5 mM; CoA, 0·255 mM; butyryl-AMP, as shown; AMP, as shown; enzyme (fraction II), 11·7 μg. of protein/ml. of reaction mixture. ○, No AMP; ▲, 1·6 mM-AMP; ●, 3·2 mM-AMP; △, 6·4 mM-AMP. Incubation was for 10 min. at 37°. v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

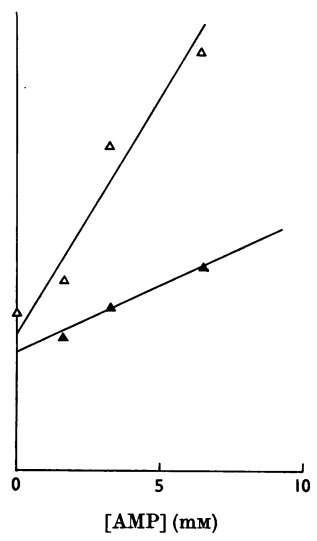


Fig. 10. Replot of slopes (△) and intercepts (▲) derived from Fig. 9 against various concentrations of AMP. Ordinate scales for (△) and (▲) are the ratio of ordinates to abscissa and the ordinate of Fig. 9 respectively.

To distinguish between the various possibilities, and to differentiate between A and B with regard to their order of entry into the reaction, product-inhibition studies were carried out. With butyryl-AMP as the variable substrate and CoA maintained at saturating concentration, AMP inhibited the reaction non-competitively (Fig. 9). The double-reciprocal plots, as well as the replots of slopes and intercepts (derived from Fig. 9) against the concentration of AMP, were linear (Fig. 10). With CoA as the variable substrate and butyryl-AMP at saturating concentration, AMP inhibited uncompetitively (Fig. 11). The double-reciprocal plots were linear and the replots of intercepts against the inhibitor concentration were likewise linear (Fig. 12). When butyryl-CoA was used as the inhibitory product, competitive inhibition was obtained when CoA served as the variable substrate and butyryl-AMP was kept constant at non-saturating concentration (Fig. 13). Replot of the slopes from Fig. 13 as a function of inhibitor concentration gave a parabola (Fig. 14). No product inhibition by butyryl-CoA was observed when butyryl-AMP was varied (from 0·145 to 1·16 mM) and CoA maintained at saturating concentration, with 4·7 μg. of fraction II/ml. of reaction mixture and inhibitor in the range 0·197–0·790 mM.

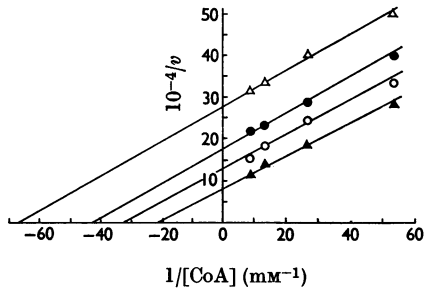


Fig. 11. Effect of AMP on double-reciprocal plots of various concentrations of CoA with butyryl-AMP at saturating concentration. The incubation mixture contained: tris–HCl buffer, pH 8·0, 70 mM; $MgCl_2$, 5 mM; butyryl-AMP, 1·35 mM; enzyme (fraction II), 10·7 μg. of protein/ml. of reaction mixture; CoA, as shown; AMP, as shown. ▲, No AMP; ○, 2 mM-AMP; ●, 4 mM-AMP; △, 8 mM-AMP. Incubation was for 10 min. at 37°. v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

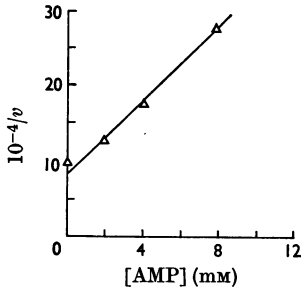


Fig. 12. Replot of intercepts derived from Fig. 11 against various concentrations of AMP.

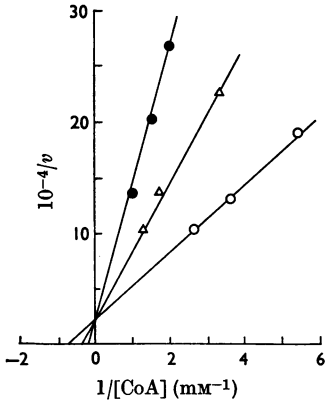
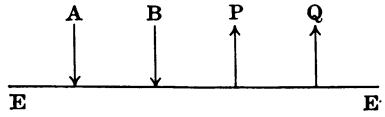


Fig. 13. Effect of butyryl-CoA on double-reciprocal plots of various concentrations of CoA with butyryl-AMP constant at non-saturating concentration. The incubation mixture contained: tris–HCl buffer, pH 8·0, 70mM; $MgCl_2$, 4mM; butyryl-AMP, 0·90mM (non-saturating); CoA, as shown butyryl-CoA, as shown; enzyme (fraction II), 5·6μg. of protein/ml. of reaction mixture. ○, 0·26mM-butyryl-CoA; △, 0·41mM-butyryl-CoA; ●, 0·54mM-butyryl-CoA. Incubation was for 10min. at 37°. v=m-moles CoA disappeared during the experiment/ml. of reaction mixture.

The results represented here fit eqn. (3), which is the rate law for the ordered Bi Bi mechanism:



where A, B, P and Q stand for CoA, butyryl-AMP, AMP and butyryl-CoA respectively.

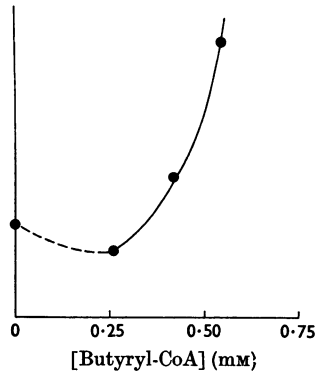


Fig. 14. Replot of slopes derived from Fig. 13 versus various concentrations of butyryl-CoA. The ordinate scale is composed of the ratios of the ordinates to the abscissa in Fig. 13.

where V_1 and V_2 are the maximum velocities of the forward and backward reactions respectively, K_a, K_b, K_p and K_q are the Michaelis constants of A, B, P and Q respectively, K_{ia}, K_{ib}, K_{ip} and K_{iq} are the inhibition constants for A, B, P and Q respectively, and $K_{eq.}$ is the equilibrium constant.

By taking reciprocals and arranging terms, equations corresponding to Figs. 9, 11 and 13 are obtained:

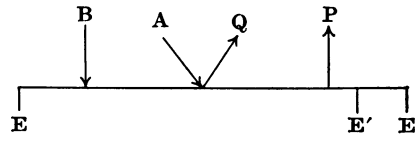
$$\frac{1}{v}=\frac{K_b}{V_1}\left(1+\frac{P}{\frac{K_pK_{iq}}{K_q}}\right)\frac{1}{B}+\frac{1}{V_1}\left(1+\frac{P}{K_{ip}}\right) \qquad (3a)$$

$$\frac{1}{v}=\frac{K_a}{V_1}\cdot\frac{1}{A}+\frac{1}{V_1}\left(1+\frac{P}{K_{ip}}\right) \qquad (3b)$$

$$\frac{1}{v}=\frac{K_a}{V_1}\left(1+\frac{Q}{K_{iq}}\right)\frac{1}{A}+\frac{1}{V_1} \qquad (3c)$$

from which the desired kinetic constants may be derived.

On the other hand, it could be shown by using the method of King & Altman (1956) that the same rate equation is obeyed by the 'Iso-Theorell–Chance' mechanism (Cleland, 1963*a*):



$$v=\frac{V_1V_2\left(AB-\frac{PQ}{K_{eq.}}\right)}{K_{ia}K_bV_2+K_bV_2A+K_aV_2B+V_2AB+\frac{K_qV_1P}{K_{eq.}}+\frac{K_pV_1Q}{K_{eq.}}+\frac{V_1PQ}{K_{eq.}}+\frac{K_qV_1AP}{K_{ia}K_{eq.}}+\frac{K_aV_2BQ}{K_{iq}}+\frac{V_2ABP}{K_{ip}}+\frac{VBPQ}{K_{ib}K_{eq.}}} \qquad (3)$$

in which the order of the A and B and the P and Q symbols is reversed and isomerization of the enzyme takes place during the reaction. Kinetic methods alone cannot differentiate between the two mechanisms and usually binding studies are required to detect which substrate enters the reaction first.

But in this case one of the two alternative mechanisms suggested for reaction (*b*) may be ruled out by considering the mechanism of the overall reaction. In the obligatory ordered combination of the three substrates with the enzyme in the overall reaction, the possibility of CoA entering first was eliminated. On the other hand, an ordered Bi Bi mechanism as suggested for reaction (*b*) compels CoA to combine with the free enzyme. (The possibility of random combination of enzyme with CoA could be ruled out because of the linearity of Figs. 1, 3 and 7.) As both the overall reaction and reaction (*b*) are catalysed by the same enzyme it could not reasonably be assumed that CoA is the first substrate in reaction (*b*) and the second substrate in the overall reaction. Therefore it is concluded that in both reactions CoA enters as the second substrate and the 'Iso-Theorell–Chance' model is the mechanism of choice for reaction (*b*). Confirmatory evidence was obtained by carrying out reaction (*b*) in the presence of pantotheine, a substrate analogue of CoA, at various butyryl-AMP concentrations. The double-reciprocal plot was linear (Fig. 15). If CoA had been the first substrate to enter reaction (*b*), the observed plot would be expected to deviate from linearity in the presence of substrate analogue (Wong & Hanes, 1962).

Butyryl-CoA activation. The 'Iso-Theorell–Chance' mechanism proposed for reaction (*b*) does not imply a parabolic pattern for the replot presented in Fig. 14. Further investigation of the butyryl-CoA effect on reaction (*b*) resulted in the demonstration of a dual butyryl-CoA effect on the system. Relatively small concentrations of butyryl-CoA activated the butyryl-AMP-dependent CoA disappearance, whereas larger amounts exerted product inhibition. Butyryl-CoA activation is illustrated in Fig. 16, where the rate of reaction, v, is plotted against increasing butyryl-AMP concentrations. CoA was kept fixed at saturating concentration, to eliminate product inhibition by butyryl-CoA. Butyryl-CoA activation could be

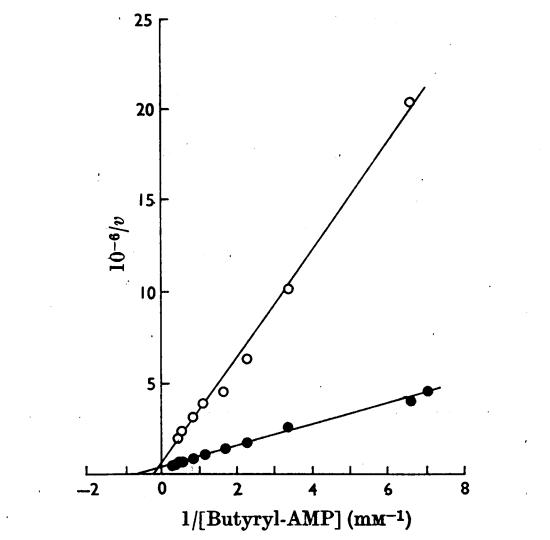


Fig. 15. Effect of pantotheine on double-reciprocal plot at various butyryl-AMP concentrations. The incubation mixture contained: tris–HCl buffer, pH 8·0, 104mM; $MgCl_2$, 15mM; enzyme (fraction II), 10·8μg. of protein/ml. of reaction mixture; CoA, 0·044mM; butyryl-AMP, as shown. ○, No pantotheine; ●, 0·485mM-pantotheine. Incubation was for 45min. at 37°. v=m-moles of CoA disappearing during the experiment/ml. of reaction mixture, corrected to 10min. incubation time.

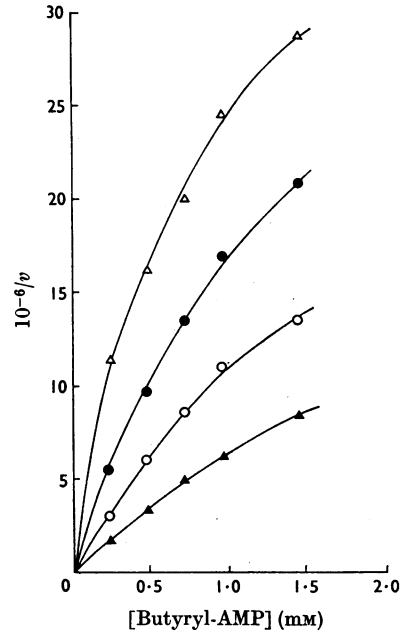


Fig. 16. Activating effect of butyryl-CoA on the butyryl-AMP-dependent CoA disappearance. The reaction mixture contained: tris–HCl buffer, pH 8·0, 75mM; $MgCl_2$, 5mM; CoA, 0·25mM; enzyme (fraction II), 11·1μg. of protein/ml. of reaction mixture; butyryl-AMP, as shown; butyryl-CoA, as shown. ▲, No butyryl-CoA; ○, 0·05mM-butyryl-CoA; ●, 0·30mM-butyryl-CoA; △, 0·43mM-butyryl-CoA. Incubation was for 10min. at 37°. v=m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

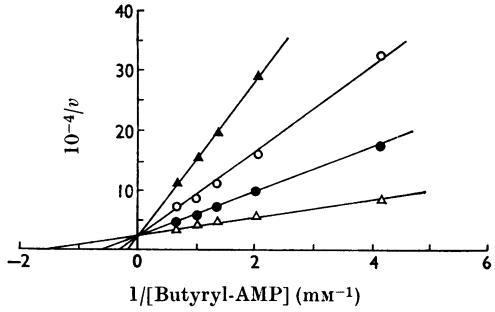


Fig. 17. Effect of butyryl-CoA on the double-reciprocal plots of various concentrations of butyryl-AMP at saturating CoA concentration. The reaction mixture contained: tris–HCl buffer, pH 8·0, 75 mM, $MgCl_2$, 5 mM; CoA, 0·25 mM; enzyme (fraction II), 11·1 μg. of protein/ml. of reaction mixture; butyryl-AMP, as shown; butyryl-CoA, as shown. ▲, No butyryl-CoA; ○, 0·05 mM-butyryl-CoA; ●, 0·30 mM-butyryl-CoA; △, 0·43 mM-butyryl-CoA. Incubation was for 10 min. at 37°. v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

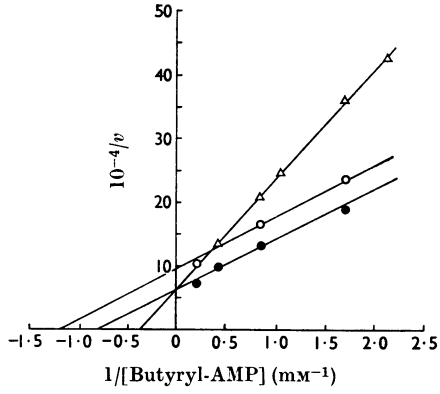


Fig. 18. Effect of ATP and butyrate on double-reciprocal plots of various concentrations of butyryl-AMP. The incubation mixture contained: tris–HCl buffer, pH 8·0, 76 mM; $MgCl_2$, 15 mM; CoA, 0·182 mM; enzyme (fraction II), 84 μg. of protein/ml. of reaction mixture; butyryl-AMP, as shown; ATP, as shown; butyrate, as shown. ●, No additions; ○, 76 mM-butyrate; △, 0·02 M-ATP. Incubation was for 10 min. at 37°. v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

abolished by saturating the system with butyryl-AMP (Fig. 17). The possibility that butyryl-CoA activates reaction (*b*) by combination with the free enzyme (competitive activation) is discussed below.

Dead-end inhibition of reaction (b) by substrates of the overall reaction. The establishment of the mechanism of reaction (*b*) enables us to decide which of the two alternative ordered sequences B–C–A and A–C–B applies in the overall reaction, by considering the effect of ATP and butyrate as dead-end inhibitors of the reaction. As ATP or butyrate may combine with the free enzyme in the overall reaction, one of them would be expected to compete with butyryl-AMP in reaction (*b*). The first substrate to enter the overall reaction will thus become a competitive inhibitor of butyryl-AMP.

The effect of butyrate and ATP on reaction (*b*) at variable butyryl-AMP concentrations is shown in Fig. 18. Only ATP was found to compete with butyryl-AMP, whereas butyrate inhibition was uncompetitive, proving the A–C–B sequence of substrates for the overall reaction.

DISCUSSION

Before the work of Webster & Campagneri (1962) the role of fatty acyl-AMP as an intermediate in the overall reaction of fatty acid activation was repeatedly questioned because of the difficulties encountered in isolating it from the reaction mixture. Despite the fact that, according to Berg (1956), fatty acyl-AMP is cleaved by either PP_i or CoA in systems activating fatty acids of different chain lengths several alternative mechanisms have been proposed in which fatty acyl-AMP is not an intermediate in the overall reaction. By measuring the rate of isotope exchange at equilibrium, with acetyl-CoA synthetase, Boyer *et al.* (1959) demonstrated that equilibrium conditions resulted in a rate of acetate incorporation equal or definitely greater than the rate of AMP incorporation into ATP. By analogy with the glutamine synthetase reaction it was suggested that CoA, acetate and ATP enter the reaction in the order specified and that acyl-AMP is not a true intermediate in the overall reaction. Similar sequential mechanisms have been suggested by Cornforth (1959) and by Ingraham & Green (1958), in contrast with Berg's (1956) Ping Pong mechanism. In all these schemes CoA is essential for the initiation of the overall reaction and becomes enzyme-bound before the first product is released.

The demonstration of enzyme-bound butyryl-AMP formed from ATP and butyric acid in the absence of CoA (Webster & Campagneri, 1962) is in confirmation of Berg's (1956) mechanism for the medium-chain acyl-CoA synthetase. Enzyme fraction I (Bar-Tana & Rose, 1968), catalysing the synthesis of butyryl-CoA, was indeed shown to operate according to Berg's (1956) scheme. On the other hand, a different mechanism for butyrate activation is indicated by kinetic study of fraction II, namely the sequential entry of substrates in the order A, C and B (standing for ATP, CoA and butyrate). With such ordered entry of substrates in the overall reaction butyryl-AMP is not an

intermediate, at least in the sense of Berg's (1956) scheme, which postulates two distinct reaction steps, where butyryl-AMP is the product of the first and the substrate for the second partial reaction. Kinetic analysis alone, however, gives no information as to the character of the quaternary complex enzyme – ATP – CoA – butyrate and its transformations until the release of the products of the reaction. Therefore it might well be that acyl-AMP as such appears bound to the enzyme in one of these transformations. In any case the complex, if formed at all, is not a 'stable' one according to Cleland's (1963*a*,*b*,*c*) definition, and the findings of Webster & Campagneri (1962) do not apply in this system.

From the sequential mechanism suggested for the overall reaction it follows that butyryl-AMP-dependent CoA disappearance is not a part of this reaction but analogous to it. The enzyme site specific for ATP may bind butyryl-AMP, presumably by the AMP moiety. Supporting evidence for this is provided by the illustration of the 'Iso-Theorell–Chance' (Cleland, 1963*a*) mechanism for the butyryl-AMP-dependent CoA disappearance, where the free enzyme combines either with butyryl-AMP or with AMP. These findings, together with the competitive relationship between butyryl-AMP and ATP, indicate that both the overall reaction and the butyryl-AMP-dependent CoA disappearance are initiated by the combination of free enzyme with the AMP moiety, resulting in the enzyme form required for combination with CoA.

With regard to the mechanism of reaction (*b*), the dual effect of butyryl-CoA deserves notice. At low concentrations, butyryl-CoA activates the butyryl-AMP-dependent disappearance of CoA, whereas at high concentrations it acts as a product inhibitor. To eliminate butyryl-CoA activation, saturating concentrations of butyryl-AMP were maintained when the product-inhibition pattern of butyryl-CoA was studied. On the other hand, by maintaining a saturating concentration of CoA conditions were established whereby butyryl-CoA activation could be studied without the interference of butyryl-CoA product inhibition. Information as to the nature of the activating effect of butyryl-CoA on reaction (*b*) was provided by the competition between butyryl-CoA and butyryl-AMP for the free enzyme. As butyryl-CoA activation occurred also at saturating concentrations of CoA, it is concluded that CoA does not have a higher affinity for the butyryl-CoA–enzyme–butyryl-AMP complex than for the enzyme–butyryl-AMP complex.

It is not yet known whether the activation effect is due to a greater affinity of the free enzyme for butyryl-AMP in the presence of butyryl-CoA, or whether the rate of the isomerization step plays a role in the activation. A mechanism for product modification similar to that described here is suggested by Wong & Hanes (1964) for horse liver alcohol dehydrogenase.

Supporting evidence for an ordered mechanism of the overall reaction is provided by the complete absence of reaction (*a*), i.e. the formation of ATP from PP_i and butyryl-AMP. If butyryl-AMP is not in intermediate in the overall reaction and CoA is necessary for PP_i release, it would be expected that in the reverse reaction PP_i would not be incorporated into ATP in the absence of CoA. Also, according to the 'Iso-Theorell–Chance' mechanism suggested for reaction (*b*), in which the ternary complex enzyme–butyryl-AMP–CoA is unstable and releases butyryl-CoA as product before PP_i cleavage of butyryl-AMP can take place, ATP cannot be formed even in the presence of CoA. This raises the question how, if at all, ATP can be formed by the reversal of the overall reaction. As the overall reaction was studied under initial-velocity conditions, the product concentrations being kept at zero, no information as to the mode of release of products could be obtained. Further, as reaction (*b*) is not a reaction step of the overall reaction, it is difficult to decide if the release of butyryl-CoA and AMP, which are the products common to both reactions, occurs in a similar fashion. However, two alternative pathways of the reversal of the overall reaction are possible:

(*c*) $\text{Butyryl-CoA} + \text{AMP} + PP_i \rightleftharpoons \text{butyryl-AMP} + \text{CoA} + PP_i$

(*d*) $\text{Butyryl-CoA} + \text{AMP} + PP_i \rightleftharpoons \text{butyrate} + \text{ATP} + \text{CoA}$

Both the relative activity and the intrinsic mechanism of the two alternative pathways will determine whether ATP will be formed from AMP, PP_i and butyryl-CoA. If, for example, reaction (*c*) is dominant, ATP formation will hardly take place.

Experiments to clarify this problem were complicated by the potent butyryl-CoA inhibition of the reaction system used to determine ATP formation, (hexokinase and glucose 6-phosphate dehydrogenase) and further studies are still required.

Linearity of the plots and replots of the kinetic data obtained with enzyme fraction II indicates the absence of allosteric behavior towards the various ligands that acted as allosteric effectors of enzyme fraction I (Bar-Tana & Rose, 1968). Taking into consideration the possibility that the two enzyme fractions can be transformed into one another, it is tempting to explain the absence of allostery in fraction II as reflecting either a dissociation step leading from fraction I to fraction II according to the MWC model (Monod,

Wyman & Changeux, 1965), or some other deterioration of fraction I protein preventing allosteric transformation.

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