Studies on Medium-Chain Fatty Acyl-Coenzyme A Synthetase

ENZYME FRACTION I: MECHANISM OF REACTION AND ALLOSTERIC PROPERTIES

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1. The mechanism of butyrate activation catalysed by an enzyme fraction derived from ox liver particles (fraction I; Bar-Tana, Rose & Shapiro, 1968) was studied by an analysis of the initial-velocity pattern of the overall reaction and found to conform to the Bi Uni Uni Bi Ping Pong model (Cleland, 1963*a,b,c*) in agreement with the reaction scheme proposed by Berg (1956). 2. A homotropic co-operative effect was exerted by CoA on fraction I, whereas ATP and AMP functioned as heterotropic co-operative ligands with respect to butyryl-AMP-dependent CoA disappearance. On the other hand, PP_i and butyryl-CoA showed antagonistic heterotropic effects when tested under similar conditions. With respect to the overall reaction CoA and ATP could be shown to function as co-operative homotropic modifiers. 3. Two interchangeable conformational states of the enzyme are therefore presumed to exist, state R, having a higher affinity for CoA and ATP and thus preferentially catalysing butyryl-AMP-dependent CoA disappearance (partial reaction *b*), and state T, favoured by the presence of PP_i, catalysing the formation of ATP from butyryl-AMP and PP_i (partial reaction *a*) with greater efficiency. 4. These findings serve to explain the opposite effects of ATP on the partial reactions, as well as the inhibition by CoA and ATP of ATP formation (reaction *a*) and by PP_i of the butyryl-AMP-dependent CoA disappearance (reaction *b*) (Bar-Tana *et al.* 1968). 5. The possible analogy of these observations to amino acid-activating and other similar systems is discussed.

As described in the preceding paper (Bar-Tana, Rose & Shapiro, 1968), two enzyme fractions I and II, capable of catalysing the activation of butyrate according to the overall reaction:

may be isolated from ox liver particles. Fraction I, the subject of the present paper, catalyses in addition the formation of ATP from PP_i and butyryl-AMP (reversal of reaction *a* below) and the butyryl-AMP-dependent disappearance of CoA (reaction *b*), whereas fraction II is incapable of catalysing the reversal of reaction *a*:

As both reactions (*a*) and (*b*) are two consecutive steps of the overall reaction according to Berg (1956), it is most likely that Berg's mechanism applies for the reaction catalysed by fraction I, whereas fraction II seems to differ in its mode of

action. To elucidate this point further, it seemed feasible to examine the reaction system kinetically and compare the findings with the Bi Uni Uni Bi Ping Pong model predictions proposed by Cleland (1963*a,b,c*) for multisubstrate and multiproduct systems.

Two observations in our previous study of this fraction are not accounted for by Berg's (1956) scheme of reaction: (*a*) the activation of butyryl-AMP-dependent CoA disappearance by ATP and (*b*) the inactivation of the ATP-forming system (reversal of reaction *a*) by prior incubation of fraction I with CoA or ATP or both. The ATP inhibition of ATP formation from butyryl-AMP and PP_i cannot be explained by product inhibition, since the inhibitory effect was demonstrated after the removal of free ATP by gel filtration. Nor can CoA inhibition be the result of competition for butyryl-AMP by the simultaneous occurrence of partial reaction (*b*), since free CoA was also removed by gel filtration before the experiment. It is unlikely that the contrasting effects exerted by ATP on the two partial reactions (*a*) and (*b*) reflect the presence of two separate enzymes catalysing

each reaction step, in view of the fact that the two partial activities could not be separated during the enzyme fractionation. On the other hand, the possibility of two different conformations of enzyme protein, catalysing each one of the two partial reactions with different efficiency, and whose transformation from one into the other would be influenced by ATP, would explain the effects observed. If this were the case, CoA and PP_i could discriminate between two assumed enzyme conformations.

In the present paper a study of the butyrate-activating enzyme in the light of the conformational aspects that might be involved is reported. The allosteric behaviour of fraction I towards different ligands that are substrates and products of the overall reaction is described. Also, it is shown by kinetic analysis of the overall reaction that the reaction mechanism fits the Bi Uni Uni Bi Ping Pong model (Cleland, 1963*a,b,c*) and is therefore in agreement with that suggested by Berg (1956).

EXPERIMENTAL

Materials, source and purification of enzyme and substrates, as well as methods of assay, were as described in the preceding paper (Bar-Tana *et al.* 1968). The free AMP present in butyryl-AMP preparation was removed by passage through a DEAE-Sephadex column (0.8 cm. × 12 cm.) equilibrated with a buffer mixture containing 100 mM-tris-HCl buffer, pH 7.4, and 45 mM-NaCl. Butyryl-AMP (anhydride/AMP ratio 1:1) emerged first, followed much later by free AMP. As butyryl-AMP is hydrolysed easily in aqueous solution, the separation from AMP was carried out just before the experiment. Butyryl-CoA was prepared by the method of Smith (1965). Redistilled butyric anhydride was treated with CoA in bicarbonate solution at equimolecular quantities at 0° for 5 min. The pH was then lowered to 3.0 and the mixture extracted three times with diethyl ether. Excess of ether was evaporated under N₂. Butyryl-CoA content was estimated by measuring the difference between total CoA (measured by absorption spectrum at 260 mμ) and free CoA (Ellman, 1958). Free CoA was removed by adding Ellman reagent to the concentrated butyryl-CoA solution and passing the mixture through a DEAE-Sephadex column (0.8 cm. × 12 cm.) prepared similarly to that used in the separation of AMP from butyryl-AMP.

After application of an elution gradient from 45 mM-NaCl to 0.5 M-NaCl the butyryl-CoA emerged first (at about 0.28 M-NaCl) and the thionitrobenzoate derivative of CoA was eluted shortly afterwards.

Kinetic measurements. Kinetic measurements were carried out of the overall reaction and reaction (b). Initial velocities were measured by choosing enzyme concentrations ensuring strictly linear relationships between incubation time and rates of reaction. Conditions of saturation or non-saturation of the enzyme by the various substrates were determined before each set of kinetic experiments.

The kinetic data were analysed by constructing double-reciprocal plots of velocity and substrate concentrations, followed by replots of slopes and intercepts against reciprocal

Fig. 1. Double-reciprocal plots of initial velocity against variable butyrate concentrations at saturating concentrations of ATP and various concentrations of CoA. The incubation mixture contained: tris-HCl buffer, pH 8.0, 100 mM; MgCl₂, 14 mM; enzyme (fraction I), 96 μg. of protein/ml. of reaction mixture; ATP, 36 mM; CoA, as shown; butyrate, as shown. Δ, 0.109 mM-CoA; ●, 0.137 mM-CoA; ○, 0.274 mM-CoA; ▲, 0.410 mM-CoA. Incubation was for 10 min. at 37°. *v* = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

concentrations of the changing fixed substrate in the initial-velocity studies, or against the inhibitor concentration in the product-inhibition studies. Single points that deviated widely from the general pattern of the double-reciprocal plots were discarded. The replots were fitted to a straight line or parabola by the least-square regression method. More complex functions were not encountered during this study. When necessary, kinetic constants were obtained directly from the replots, though this was not done in all cases. Conclusions on the mechanism of the reaction were derived from the shape of the reciprocal plots and replots and not by comparison of the kinetic constants with expected Haldane relationships obtained from the different sets of kinetic measurements.

RESULTS

Initial-velocity studies of the overall reaction. Confirmation of Berg's (1956) reaction mechanism for the activation of butyrate catalysed by fraction I may be obtained by studies of the initial velocity of the overall reaction, and by comparing the kinetic pattern obtained with that of the Bi Uni Uni Bi Ping Pong model.

Initial-velocity studies of the overall reaction were carried out at different concentrations of two

Fig. 2. Replot of intercepts derived from Fig. 1 against the reciprocal of the concentration of CoA.

Fig. 3. Double-reciprocal plots of initial velocity against variable butyrate concentrations at saturating concentrations of CoA and various concentrations of ATP. The incubation mixture contained: tris-HCl buffer, pH 8.0, 100 mM; MgCl_2 , 14 mM; enzyme (fraction I), 96 μg . of protein/ml. of reaction mixture; CoA, 0.17 mM; butyrate, as shown; ATP, as shown. $\circ$, 4 mM-ATP; $\blacktriangle$, 8 mM-ATP; $\bullet$, 16 mM-ATP. Incubation was for 10 min. at 37° . v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

of the three substrates, with the third one kept constant. With sodium butyrate as the variable substrate, at several fixed concentrations of CoA and ATP at saturating concentration, the double-reciprocal plots were linear and constituted a family of parallel lines (Fig. 1). Replots of the intercepts

Fig. 4. Replot of slopes ($\bullet$) and intercepts ($\times$) derived from Fig. 3 against the reciprocal of the concentration of ATP. The ordinate scales for ($\bullet$) and ($\times$) are the ratios of the ordinates to abscissa and the ordinates in Fig. 3 respectively.

Fig. 5. Double-reciprocal plots of initial velocity against variable concentrations of CoA at fixed near-saturating concentrations of butyrate and various concentrations of ATP. The incubation mixture contained: tris-HCl buffer, pH 8.0, 100 mM; MgCl_2 , 14 mM; enzyme (fraction I), 78.5 μg . of protein/ml. of reaction mixture; ATP, as shown; CoA, as shown; butyrate, 71 mM. Δ , 6.9 mM-ATP; $\bullet$, 8.65 mM-ATP; $\circ$, 10.3 mM-ATP; $\blacktriangle$, 12.1 mM-ATP; $\times$, replot (Fig. 7). Incubation was for 10 min. at 37° . v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

against the reciprocal of the concentration of CoA were also linear (Fig. 2). With butyrate as the variable substrate at several fixed concentrations of ATP and with CoA at saturating concentration, the double-reciprocal plots were again linear, constituting a family of lines intersecting to the left of the vertical axis (Fig. 3). Replots of slopes and intercepts against the reciprocal of the concentration of ATP were also linear (Fig. 4). With CoA

Fig. 6. Double-reciprocal plots of initial velocities against variable ATP concentrations at several CoA concentrations, with butyrate constant at near-saturating concentration. Experimental conditions were as given in Fig. 5. —, Replot of intercepts from Fig. 5; ○, 0.0505 mM-CoA; ▲, 0.076 mM-CoA; ●, 0.101 mM-CoA; △, 0.152 mM-CoA. v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture.

Fig. 7. Replot of intercepts derived from Fig. 6 against the reciprocal of the concentration of CoA.

as the variable substrate, at several fixed concentrations of ATP and with butyrate constant at saturating concentration, the double-reciprocal plots tended to deviate from linearity and developed into parabolas. The deviation was more pronounced at low ATP concentrations, whereas at relatively higher ATP concentrations the parabolic double-reciprocal plots degenerated into a family of almost straight parallel lines (Fig. 5). The same pattern was obtained at non-saturating concentrations of sodium butyrate. On interchanging ATP and CoA in the double-reciprocal plots, the parabolic pattern became even more evident at low CoA concentrations (Fig. 6). Replotting the intercepts derived from Fig. 6 against reciprocal CoA concentrations

resulted in a straight line (Fig. 7), demonstrating that at saturating concentrations of ATP the degeneration of the parabolic pattern obtained in Fig. 5 is complete.

Except for the deviations from linearity at low ATP concentrations just described and discussed below, the pattern observed in Figs. 1–7 fits eqn. (1), which is the initial-velocity law for the Bi Uni Uni Bi Ping Pong mechanism:

$$v = \frac{VABC}{K_{ia}K_bC + K_cAB + K_bAC + K_aBC + ABC} \quad (1)$$

where V is the maximum velocity of the forward reaction, A , B and C are the concentrations of substrates A , B and C , K_a , K_b and K_c are the Michaelis constants of substrates A , B and C respectively, and K_{ia} , K_{ib} and K_{ic} are the inhibition constants for A , B and C respectively.

Assuming A , B and C to stand for ATP, butyrate and CoA respectively, Figs. 1, 3 and 5 fit eqns. (1a), (1b), and (1c) respectively, derived from the rate law (1) by rearranging terms and adapting to corresponding experimental conditions:

$$\frac{1}{v} = \frac{K_b}{VB} + \frac{1}{V} \left(1 + \frac{K_c}{C} \right) \quad (1a)$$

$$\frac{1}{v} = \frac{K_b}{V} \left(1 + \frac{K_{ia}}{A} \right) \frac{1}{B} + \frac{1}{V} \left(1 + \frac{K_a}{A} \right) \quad (1b)$$

$$\frac{1}{v} = \frac{K_c}{VC} + \frac{1}{V} \left(1 + \frac{K_a}{A} \right) \quad (1c)$$

By interchanging A and B , so that B becomes the first substrate to enter the reaction, the same kinetic pattern is obtained. It should be pointed out, however, that the initial-velocity law described by eqn. (1) fits also the Bi Bi Uni Uni Ping Pong model:

Again, by assuming A , B and C to stand for ATP, butyrate and CoA respectively, equations equivalent to (1a), (1b), and (1c) may be derived. Discrimination between these two alternative mechanisms is only possible by product inhibition studies, by $AMP \rightleftharpoons$ butyryl-AMP exchange in the presence or absence of CoA, or by initial-velocity studies of the partial reaction (b). However, in view of the isolation of a 'stable' enzyme-bound butyryl-AMP complex (Webster & Campagneri, 1962), the

Fig. 8. Co-operative effects of CoA and ATP on butyryl-AMP-dependent CoA disappearance. The incubation mixture contained: tris-HCl buffer, pH 8.0, 100mM; MgCl_2 , 14mM; enzyme (fraction I), 24 μg . of protein/ml. of reaction mixture; CoA, as shown; ATP, as shown; butyryl-AMP, 1.4mM. $\bullet$, Without modifier; $\circ$, +36mM-ATP; Δ , +42mM-ATP. Incubation was for 10min. at 37° . v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture. (a) Direct plot; (b) double-reciprocal plot.

Bi Bi Uni Uni Ping Pong mechanism becomes a less likely model for the reaction catalysed by fraction I. Sequential Ter Ter and Hexa Uni Ping Pong mechanisms could be ruled out because of the kinetic picture obtained.

The deviation from linearity shown in the double-reciprocal plots of Figs. 5 and 6 at low CoA and ATP concentrations is not expected by the analysis of the initial-rate law for the Bi Uni Uni Bi Ping Pong model. The parabolic pattern obtained may indicate allosteric behaviour of the enzyme protein towards ATP at low CoA concentrations, and towards CoA at low ATP concentrations, whereas near-saturating concentrations of one of the allosteric ligands

Fig. 9. Co-operative effects of CoA and AMP on butyryl-AMP-dependent CoA disappearance. The incubation mixture contained: tris-HCl buffer, pH 8.0, 100mM; MgCl_2 , 14mM; enzyme (fraction I), 36 μg . of protein/ml. of reaction mixture; CoA, as shown; AMP, as shown; butyryl-AMP, 1.67mM. $\times$, Without modifier; $\blacktriangle$, +6mM-AMP; Δ , +18mM-AMP; $\bullet$, +36mM-AMP. Incubation was for 10min. at 37° . v = m-moles of CoA disappearing during the experiment/ml. of reaction mixture. (a) Direct plot; (b) double-reciprocal plot.

abolished the co-operative effect of the other. Sodium butyrate does not participate in the behaviour described, as neither saturating nor non-saturating concentrations of sodium butyrate affected the parabolic pattern of the double-reciprocal plots.

Studies of partial reaction (b). The conformational change induced by ATP and CoA, indicated in the initial-velocity studies of the overall reaction, was

Fig. 10. Inhibitory effects of butyryl-CoA and PP_i on the butyryl-AMP-dependent CoA disappearance. Experimental conditions were as given in Fig. 9. $\times$, Without modifier; Δ , +0.16 mM- PP_i ; $\circ$, +0.8 mM- PP_i ; $\bullet$, +0.1 mM-butyryl-CoA.

Fig. 11. Heterotropic co-operative effects of butyryl-CoA and PP_i on the butyryl-AMP-dependent CoA disappearance as revealed by the presence of AMP. Experimental conditions were as given in Fig. 9. $\times$, +18 mM-AMP; $\bullet$, +0.4 mM- PP_i + 18 mM-AMP; Δ , +0.1 mM-butyryl-CoA + 18 mM-AMP; $\circ$, +0.16 mM- PP_i ; $\blacktriangle$, +0.1 mM-butyryl-CoA.

further confirmed by study of the partial reaction (b) as catalysed by fraction I. As shown in Fig. 8(a), the plot of initial velocity against variable CoA concentrations deviated distinctly from the

Michaelis-Menten hyperbolic pattern. The homotropic co-operative effect of CoA was demonstrated by the sigmoidal shape of the plot of v versus $[S]$ as well as by the parabolic double-reciprocal plot shown in Figs. 8(a) and 8(b).

The heterotropic co-operative effect of ATP, already indicated by the kinetic pattern of the initial-velocity studies of the overall reaction, was confirmed for the partial reaction (b), where the co-operative interaction of CoA, represented by the sigmoidal shape of the plot of v versus $[S]$, could be converted into a Michaelis-Menten hyperbolic curve in the presence of ATP. As shown by Fig. 8(a), there is no further ATP activation of partial reaction (b) in the presence of saturating concentrations of CoA, whereas at certain low CoA concentrations no reaction occurs in the absence of ATP. This is in agreement with our earlier observation that at near-saturating concentrations of either CoA or ATP the allosteric effects in the overall reaction are abolished.

Activation of partial reaction (b) could also be demonstrated with AMP at similar concentrations to those described for ATP activation. As illustrated by Fig. 9, AMP activation is shown to be allosteric in nature, similar to that of ATP.

On the other hand, butyryl-CoA and PP_i showed antagonistic heterotropic effects, strongly inhibiting the partial reaction (b) at relatively low concentrations (Fig. 10). Since at low substrate (CoA) concentrations the co-operative effect of the inhibitor was scarcely detectable, reaction (b) was carried out in the presence of AMP (as allosteric activator) in addition to the inhibitor. As may be seen from Fig. 11, the addition of AMP revealed the allosteric effect of the inhibitor.

Butyrate, butyryl-AMP and Mg^{2+} were found not to function as allosteric effectors in the partial reaction (b).

DISCUSSION

The agreement between the kinetic analysis of the overall reaction and the Bi Uni Uni Bi Ping Pong mechanism confirmed that the activation of butyrate catalysed by fraction I proceeds according to the mechanism postulated by Berg (1956). This mode of action was expected initially, as the enzyme fraction was shown to catalyse the two discrete reaction steps (a) and (b). Thus the isolation of butyryl-AMP bound to the enzyme from incubation mixtures containing ATP and butyrate, in the absence of CoA, reported by Webster & Campagneri (1962) presumably reflects the presence of enzyme fraction I in enzyme prepared by the method of Mahler, Wakil & Bock (1953).

To discuss the allosteric behaviour of the enzyme protein the predictions of the MWC model (Monod,

Wyman & Changeux, 1965) will be applied. This latter model was chosen as an example, not by virtue of its greater merit over other models such as those of Koshland, Némethy & Filmer (1966) or Atkinson, Hathaway & Smith (1965), but because the co-operative effects observed correspond to those of the K-system of the MWC model, where both the substrate and the effector have differential affinities towards the proposed T and R states.

Assuming the homotropic co-operative effect of CoA to represent transformation of the T into the R state, activation of reaction step (b) by ATP or AMP and inhibition by PP_i or butyryl-CoA would reflect a change in the ratio (R)/(T) due to the heterotropic ligands. Thus in the presence of ATP or AMP an increase in the ratio (R)/(T) occurs, resulting in an activation effect, most pronounced at low CoA concentrations, whereas the presence of PP_i or butyryl-CoA decreases the ratio (R)/(T), leading to inhibition of the partial reaction (b). The change in the (R)/(T) ratio as a function of the allosteric ligands could be established in this study by observing the pattern of the rate-concentration curves or their respective double-reciprocal plots. Thus the sigmoid curve obtained in the presence of substrate (CoA) alone was converted into a rectangular hyperbola by the addition of activator (ATP or AMP), whereas the addition of inhibitor (PP_i or butyryl-CoA) enhanced the sigmoidal pattern. Also, the co-operativity of the inhibitor could be increased either by raising the concentration of the substrate or by the addition of activator, in further confirmation of the predictions of the MWC model. The allosteric activation of ATP of reaction step (b) accounts for the effect of ATP in this partial reaction, described in the preceding paper (Bar-Tana *et al.* 1968). Similarly the inhibition of reaction step (b) by PP_i, too potent to be accounted for by competition for butyryl-AMP of the ATP-forming step (a) with partial reaction (b), may now be explained in the light of the described allosteric transitions.

The conformational changes of the enzyme protein deduced from studies of partial reaction (b) were confirmed by kinetic analyses of the initial-velocity studies of the overall reaction. CoA, at non-saturating concentrations of ATP, as well as ATP at non-saturating concentrations of CoA, caused allosteric transition as shown by the parabolic pattern of the double-reciprocal plots. The prediction of the MWC model's K-system, that an allosteric effector will exhibit co-operative homotropic interactions, was thus confirmed for ATP with respect to the overall reaction (its effect in the partial reaction b could only be studied in the heterotropic sense). The heterotropic effect of ATP in the overall reaction could be shown by the degeneration of the parabolic double-reciprocal

plots versus CoA concentration with increasing ATP concentrations until at saturating concentration of ATP these plots were strictly linear.

As both CoA and ATP exerted allosteric effects on the overall reaction, apart from their role as substrates, care had to be taken to eliminate as much as possible the allosteric interactions in the initial-velocity measurements to evaluate their agreement with the Bi Uni Uni Bi Ping Pong mechanism. This was accomplished by maintaining ATP at saturating concentration whenever variable CoA and butyrate concentrations were used, or by maintaining CoA at saturating concentration whenever variable amounts of ATP and butyrate were used. Under these conditions the Michaelis-Menten range was preserved and Cleland's (1963*a,b,c*) propositions could be applied. Allosteric effects could not be prevented when variable concentrations of CoA and ATP were used, but these were minimized by maintaining the changing fixed substrate at near-saturating concentration.

From a comparison of the allosteric manifestations of the overall reaction and partial reaction (b) it appears that the same conformational state (R), showing a greater affinity for CoA, catalyses both the overall reaction and partial step (b), whereas the T-configuration, favoured in the presence of PP_i, seems to catalyse the ATP-forming step (reversal of partial reaction a). As either CoA or ATP increase the (R)/(T) ratio, it follows that both will inhibit ATP formation by transforming the enzyme into a configurational state incapable or poorly capable of catalysing this reaction step. Allosteric transition thus accounts for the inhibition by both CoA and ATP of the ATP-forming reaction step, as reported in the preceding paper (Bar-Tana *et al.* 1968).

Acyl activation by a mechanism involving acyl-AMP as an intermediate is typical of many biological systems, such as amino acid activation, light-emission by luciferase, pathways in panthothenic acid synthesis, and lipoic acid, fatty acid and biotin activations. Certain common features of these systems may be noted, and the findings reported in the present study may serve to shed some light on observations obtained with other activating systems.

With regard to the luciferase reaction, ATP is reported (McElroy, DeLuca & Travis, 1967) to activate the transfer of hydroxyluciferyl-AMP to CoA, similar to the ATP activation of partial reaction (b) reported in the present study. This activation becomes even more important in view of the fact that ATP is expected to inhibit this reaction step by causing hydrolysis of enzyme-bound luciferyl-AMP as described by DeLuca & McElroy (1965). Though a homotropic ATP effect has not yet been shown in the overall luciferase reaction, and

CoA apparently does not behave as an allosteric ligand (Airth, Rhodes & McElroy, 1958), the possibility of conformation changes taking place during luciferase catalysis is indicated by the relationship between pH, temperature, substrate analogues and light-emission, as well as by the optical-rotatory-dispersion spectrum and isotopic hydrogen exchange in the presence and absence of substrates (McElroy *et al.* 1967).

In the field of amino acid activation for protein biosynthesis, studies on the role of t-RNA* in the first activation step may be considered. Thus Loftfield & Eigner (1965) showed that oxidized t-RNA, though unable to serve as an acceptor for isoleucyl-AMP, could nevertheless stimulate hydroxamate formation in the isoleucyl-t-RNA synthetase system from *Escherichia coli*. As a titration for t-RNA was not presented, it is difficult to evaluate possible allosteric implications in this case. However, the analogous roles of t-RNA and CoA in amino acid and fatty acid activation respectively point to a similar function of t-RNA to that of CoA as a possible allosteric modifier.

Supporting evidence for t-RNA stimulation of the activation step of amino acids is available from studies of the t-RNA effect on the $[^{32}\text{P}]\text{PP}_i \rightleftharpoons \text{ATP}$ exchange (Hele, 1961; Ravel, Wang, Heynemeyer & Shive, 1965; Mitra & Mehler, 1966). In some systems no exchange occurs in the absence of t-RNA. It must be pointed out, however, that the study of $[^{32}\text{P}]\text{PP}_i \rightleftharpoons \text{ATP}$ exchange is accompanied by an intrinsic difficulty with regard to the evaluation of the t-RNA effect on the activation step. If an analogy exists between the amino acid- and fatty acid-activating systems, PP_i induces an allosteric transition opposite to that of ATP. As both ATP and PP_i are usually present in the exchange system, the t-RNA effect may be masked by transition due to ATP, while being reversed by the presence of PP_i . Thus the net effect of t-RNA will depend on the relative PP_i and ATP concentrations, and its conformational effect, if present at all, is likely to be inconsistent. Hence it would be preferable to employ either the method of Loftfield & Eigner (1965) (hydroxamate formation in the presence of modified t-RNA) or ATP formation by the reversal of the activating step, when testing the effect of t-RNA as allosteric modifier. Conformational changes in t-RNA structure may also influence the allosteric transitions (Hele & Barth, 1966), and should be taken into consideration when an analogy is being established between the two activating systems.

Webster (1965) has shown that the molecular weight of the catalytic unit of acetyl-CoA syn-

thetase is 35 000, whereas the molecular weight as calculated from the concentration of acetyl-AMP needed to saturate the enzyme is estimated to be about 85 000 (Webster, 1963). These values are in agreement with the sedimentation coefficient. It seems therefore likely that the difference in molecular weight is due to aggregation of enzyme molecules. Thus transition between different states, promoted by substrates of the overall reaction or by other metabolites, may occur and allosteric effects become apparent.

The activation of palmitoyl-CoA synthetase reported by Bar-Tana & Shapiro (1964) and confirmed by Rao & Johnston (1967) may be due to allosteric transition or to a mechanism of reaction different to that proposed by Berg (1956).

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*Abbreviation: t-RNA, transfer RNA.