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The Role of Biotin-Dependent Carboxylations in Biosynthetic Reactions

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THE THIRD JUBILEE LECTURE

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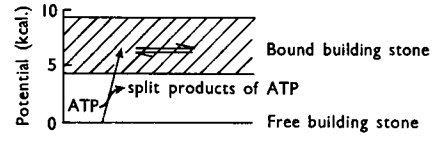
First of all I would like to express my deep gratitude to The Biochemical Society for inviting me to present the third Jubilee Lecture. This is a great honour for me, especially since The Biochemical Society is the oldest scientific society devoted exclusively to the promotion of biochemistry. Today The Biochemical Society can review 55 successful years in the advancement of the discipline. It is amusing that the inauguration date coincides almost exactly with the date of birth of this year's Jubilee lecturer; this means that The Biochemical Society and I are practically of the same age. To be precise, I arrived one month later.

At this particular time it is very interesting to recall the status of biochemistry in 1911. The elucidation of the chemical structure of natural products was well advanced, but very little was known about the dynamic aspects of the biochemical reactions. This may be illustrated appropriately by the example of alcoholic fermentation. Before 1911 it was known through the classical experiments of Harden and Young that phosphate is essential for the fermentation process, that fructose 1,6-diphosphate is formed and that a thermostable coferment participates in the fermentation process. It was not until 1911 that the enzymic splitting of pyruvate into carbon dioxide and acetaldehyde was found by Neuberg, contributing to another important landmark in the illumination of fermentation.

If one compares this status of knowledge of alcoholic fermentation and biochemistry in general with that of today, one recognizes the tremendous progress accomplished in the subsequent 55 years. Today we must admire those men of vision who founded The Biochemical Society in anticipation of this progress.

Turning to the actual theme of my lecture I should like to make a few general comments about biosynthetic processes. One of their characteristics is to synthesize complex organic molecules from simple precursors, supplied either from the diet as such or formed by the degradation of dietary components. Related to the biosynthesis of proteins, nucleic acids, polysaccharides and lipids is the requirement of energy. The living cell therefore carries out the biosyntheses by using 'activated' building stones that possess the necessary energy for the anabolic reactions. The activation is the result of chemical binding of the building stone to a carrier. In such a state transfer of the chemical moiety from a donor molecule to an acceptor molecule is possible without expenditure of additional energy. One of the first to comprehend the great significance of this class of reactions was Fritz Lipmann (1941), who introduced the term 'group transfer'.

The purpose of many catabolic mechanisms is to provide activated groups that can then be incorporated into complex molecules. In the special case when a free building stone enters the cell it must first be activated by ATP. The building stone is thus lifted to a higher potential and then becomes available for 'group transfer' reactions. The events may be illustrated by the general energy scheme shown in Scheme 1. In the lower part of the Scheme is given a selection of such 'activated'



Building stone	Carrier	Substances synthesized
Glucose	ADP	Polysaccharides
Glucose	UDP	
Choline phosphate	CMP	Phosphatides
Nucleotide	PP_i	Nucleic acids
Aminoacyl	s-RNA	Proteins

Scheme 1. General energy scheme of biosynthesis.

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building stones that are used in the biosynthesis of polysaccharides, phosphatides, nucleic acids and proteins.

In the last few years it was found that bicarbonate also can be used as a carrier in biosynthetic reactions. In these processes bicarbonate is first introduced into an acceptor molecule with the generation of a carboxylic acid, according to the following scheme:

$$AH + HCO_3^- \xrightarrow{\text{Energy}} A \cdot CO_2^- + H_2O \quad (1)$$

The incorporation of carbon dioxide into the acceptor requires energy and can be driven by the splitting of ATP into ADP and inorganic phosphate, as in carboxylation reactions:

$$AH + HCO_3^- + ATP^{4-} \rightleftharpoons A \cdot CO_2^- + ADP^{3-} + HPO_4^{2-} + H^+ \quad (2)$$

or by the decarboxylation of a carbon dioxide donor, as in transcarboxylation reactions:

$$AH + D \cdot CO_2^- \rightleftharpoons A \cdot CO_2^- + DH \quad (3)$$

The first enzyme catalysing a carboxylation reaction of this type was characterized in Ochoa's Laboratory during studies on the biological oxidation of propionic acid by way of propionyl-CoA (for review see Kaziro & Ochoa, 1964). Propionyl-CoA is carboxylated in the presence of propionyl-CoA carboxylase to form methylmalonyl-CoA, which then is isomerized into succinyl-CoA, thereby entering the citric acid cycle. Soon afterwards β-methylcrotonyl-CoA carboxylase (Lynen, 1957), acetyl-CoA carboxylase (Wakil, 1958), pyruvate carboxylase (Utter & Keech, 1960) and geranoyl-CoA carboxylase (Seubert, Fass & Remberger, 1963) were found. It can be assumed that still more enzymes of this type remain to be discovered.

Table 1. *Biotin-dependent enzymes and their substrates*

Enzyme	Substrate
Acetyl-CoA carboxylase	$CH_3 \cdot CO \cdot SCoA$
Propionyl-CoA carboxylase	$CH_3 \cdot CH_2 \cdot CO \cdot SCoA$
β-Methylcrotonyl-CoA carboxylase	$CH_3 \cdot C(CH_3):CH \cdot CO \cdot SCoA$
Geranoyl-CoA carboxylase	$R \cdot C(CH_3):CH \cdot CO \cdot SCoA$*
Pyruvate carboxylase	$CH_3 \cdot CO \cdot CO_2H$
Methylmalonyl-CoA–pyruvate transcarboxylase	$CH_3 \cdot CO \cdot CO_2H$ or $CH_3 \cdot CH_2 \cdot CO \cdot SCoA$

* $R = CH_3 \cdot C(CH_3):CH \cdot CH_2 \cdot CH_2$

All these enzymes catalyse reactions in which, with one exception, a CoA ester participates (Table 1). In all these substrates a hydrogen atom is activated by the carbonyl group of a thio ester or ketone that is adjacent to it or separated from it by a vinyl group.

Studies with β-methylcrotonyl-CoA carboxylase furnished a more intimate insight into the chemistry of the enzymic process. This enzyme, which participates in the biological degradation of leucine (Bachhawat, Robinson & Coon, 1955, 1956) by way of isovaleryl-CoA and its dehydrogenation product, could easily be isolated from micro-organism grown on isovaleric acid as sole carbon source, and was purified in our Laboratory (Lynen *et al.* 1961; Himes, Young, Ringelmann & Lynen, 1963). It was shown that the enzyme contains biotin as prosthetic group covalently bound to the protein, and that during the carboxylation a carboxybiotin enzyme intermediate is formed (Lynen *et al.* 1961; Himes *et al.* 1963; Knappe, Wenger & Wiegand, 1963). The carboxylation is thus achieved in two steps as follows:

$$ATP + HCO_3^- + \text{biotin enzyme} \xrightleftharpoons{Mg^{2+}} \text{carboxybiotin enzyme} + ADP + P \quad (4)$$

$$\text{Carboxybiotin enzyme} + CH_3 \cdot C(CH_3):CH \cdot CO \cdot SCoA\ (AH) \rightleftharpoons \text{biotin enzyme} + CH_2 \cdot CO_2H \cdot C(CH_3):CH \cdot CO \cdot SCoA\ (A \cdot CO_2H) \quad (5)$$

With these investigations the mechanism of action of biotin could finally be elucidated. That biotin plays some role in carbon dioxide fixation had long been suspected (Lardy & Peanasky, 1953).

Biotin was isolated by Kögl & Tönnis (1936) as one of the components of the 'bios' factor required by yeast for normal growth, and was later found by György, Melville, Burk & Du Vigneaud (1940) to be identical with 'vitamin H', which was known to protect against the toxicity of raw egg white. Egg white contains the protein avidin, which forms a stoicheiometric complex with biotin (Eakin, Snell & Williams, 1940, 1941). Since the complex cannot be split by the enzymes of the digestive tract, biotin deficiency develops as the result of the ingestion of raw egg white. Avidin is now generally used as a diagnostic tool of detection of biotin-dependent enzyme reactions because it inhibits all biotin enzymes. This specific inhibition technique, introduced by Wessman & Werkman (1950), was also of great value in our investigations.

To prove the reaction sequence of β-methylcrotonyl-CoA carboxylation we first used exchange

experiments with labelled substrates (Lynen *et al.* 1961). When β-methylglutaconyl-CoA labelled with ^{14}C in positions 1, 3 and 5 is incubated with unlabelled β-methylcrotonyl-CoA and the enzyme, it gives rise to labelled β-methylcrotonyl-CoA by the repeated shuttling of reaction (5) between carboxybiotin enzyme and biotin enzyme. It could be demonstrated that the enzyme-bound biotin participates because the exchange reaction was strongly inhibited by avidin. Whereas Mg^{2+} was an essential constituent of the overall carboxylation reaction, it was not required for the exchange reaction between β-methylglutaconyl-CoA and β-methylcrotonyl-CoA. Therefore one could conclude that the metal ion requirement of the overall process is limited to the first reaction step (eqn. 4), which results in the formation of carboxybiotin enzyme from bicarbonate with the expenditure of phosphate energy provided by ATP.

This ATP-dependent reaction step was also studied by the exchange technique (Lynen *et al.* 1961). In this case the exchange reactions between ATP and [^{32}P]orthophosphate or [^{14}C]ADP were measured. In agreement with eqn. (4) it was found that both exchange reactions required the simultaneous addition of the four substrates, ATP, ADP, inorganic phosphate and bicarbonate, besides the enzyme.

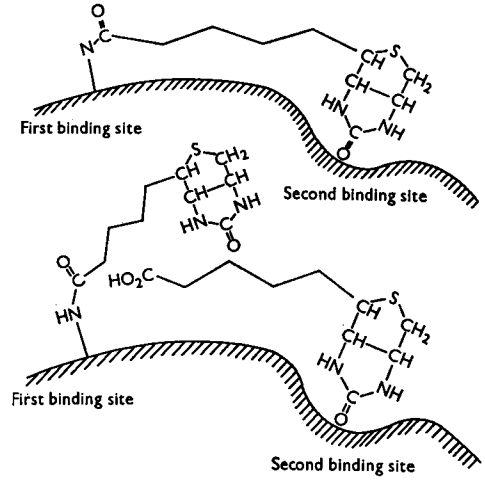
In the next few years the general validity of the sequence of reactions was proved for the biotin-dependent carboxylases of different specificity. In these investigations, in which several Laboratories participated, analogous exchange experiments were used (for review see Kaziro & Ochoa, 1964). In addition, it was possible to isolate the carboxybiotin enzymes themselves (Himes *et al.* 1963; Knappe *et al.* 1963; Kaziro & Ochoa, 1961; Lane & Lynen, 1963; Wood, Lochmüller, Riepertinger & Lynen, 1963*b*; Numa, Ringelmann & Lynen, 1964; Scrutton, Keech & Utter, 1965). This important objective was first achieved by Kaziro & Ochoa (1961) with propionyl-CoA carboxylase that had been isolated in crystalline form from pig heart. The methods used in these studies consisted in preparing the carboxybiotin enzyme either in the forward reaction (eqn. 4) by incubating the enzyme with ATP and $NaH^{14}CO_3$ in the presence of Mg^{2+}, or from the reverse of the transcarboxylation step (eqn. 5) by incubating the enzyme with the ^{14}C-labelled carbon dioxide donor. The carboxylated enzyme was then separated from the low-molecular-weight compounds by passage through a column of Sephadex or Dowex 1.

After having established the intermediate formation of carboxybiotin enzyme by the exchange experiments we turned our efforts to the elucidation of the linkage of carbon dioxide with the biotin of the enzyme. We could solve this problem through a fortunate property of β-methylcrotonyl-CoA carboxylase. It was found that free biotin, when present in high concentrations, can replace β-methylcrotonyl-CoA as substrate for the carboxylase, leading to the formation of carboxybiotin according to the following equation (Lynen *et al.* 1961):

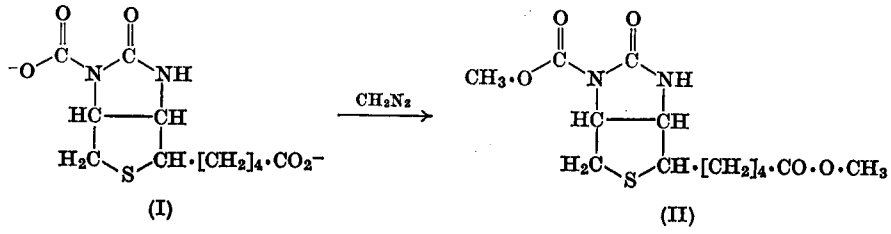
$$\mathrm{ATP} + \mathrm{HCO_3^-} + \mathrm{biotin} \xrightarrow{Mg^{2+}} \mathrm{carboxybiotin} + \mathrm{ADP} + \mathrm{P_i} \quad (6)$$

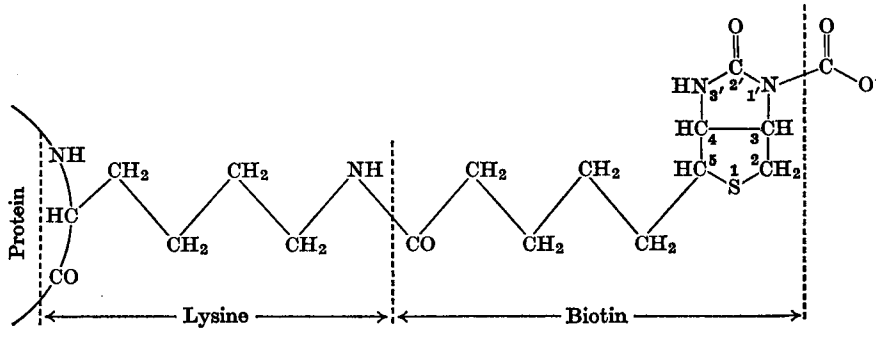
To explain this reaction, we assumed that the carboxylation of added free biotin is achieved by the partial dislocation of the enzyme-bound biotin. This seems to be possible if the biotin of the carboxylase is bound to the protein, not only by a covalent linkage through its side-chain carboxyl group, but also by a dissociable linkage between the ring system and a second site on the protein (Scheme 2). The second site also might have affinity for free biotin, and, in the presence of a large excess of the latter, the enzyme-bound biotin is partially displaced and free biotin is carboxylated. If this assumption was correct the carboxybiotin formed should be a precise model of the carboxybiotin enzyme.

In experiments with [^{14}C]bicarbonate [^{14}C]-carboxybiotin was produced. The carboxylated biotin was found to be very unstable, especially at acid pH. We were not too surprised by this feature, because for chemical reasons we had expected the imidazolidone nucleus of biotin to be the active site. It was a purely chemical problem then to stabilize the labile radioactive carboxybiotin (I) by methylation with diazomethane and to identify

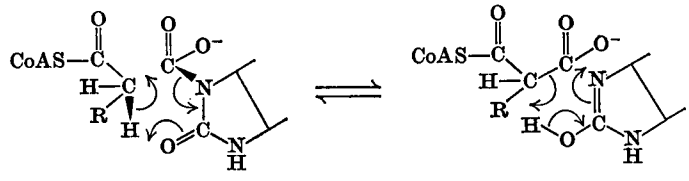


Scheme 2. Proposed mechanism for the binding of free biotin by the enzyme.





Scheme 3. Chemical structure of carboxybiotin enzyme.



Scheme 4. Mechanism of transcarboxylation.

the methylation product by comparison with authentic compounds such as 1′-*N*-carboxymethylbiotin methyl ester (II) (Lynen *et al.* 1961; Knappe, Ringelmann & Lynen, 1961).

In further studies Knappe *et al.* (1963) confirmed the prediction that this same linkage would occur also in the carboxylated enzyme itself. There 1′-*N*-carboxybiotin is bound to the enzyme protein through amide linkage at the ε-amino group of a lysine residue (Scheme 3). This was to be expected, since this mode of attachment was long recognized in biocytin, the biotin conjugate isolated from yeast by Wright *et al.* (1952). The other biotin enzymes so far investigated, namely propionyl-CoA carboxylase (Lane & Lynen, 1963), acetyl-CoA carboxylase (Numa *et al.* 1964), pyruvate carboxylase (Scrutton *et al.* 1965) and methylmalonyl-CoA–oxaloacetate transcarboxylase (Wood *et al.* 1963*b*), also contain biocytin and bind carbon dioxide in the same manner.

The chemical reactivity of an 'active carbonic acid' of this structure derives from the electron attraction of the ureido system, i.e. the cyclic urea moiety of biotin behaves like a weak acid. The carboxy derivatives of such a structure are to some extent comparable with acid anhydrides. The bond between the nitrogen atom and carbon dioxide is polarized, which augments the electrophilic character of the carboxyl group and therefore its ability to enter transcarboxylation reactions (Knappe & Lynen, 1963). In experiments with propionyl-CoA carboxylase Rétey & Lynen (1965) and Arigoni, Lynen & Rétey (1966) have shown that the transcarboxylation occurs with strict stereospecificity. In the carboxylation process that leads to the formation of *S*-methylmalonyl-CoA with retention of the steric configuration, this means that the entering carboxyl group occupies the same place that the hydrogen atom had vacated. These results agree with an electrophilic transcarboxylation mechanism. The process was found to be reversible and may occur by way of a concerted cyclic process, as depicted in Scheme 4.

The reactivity of the biotin-bound carbonic acid

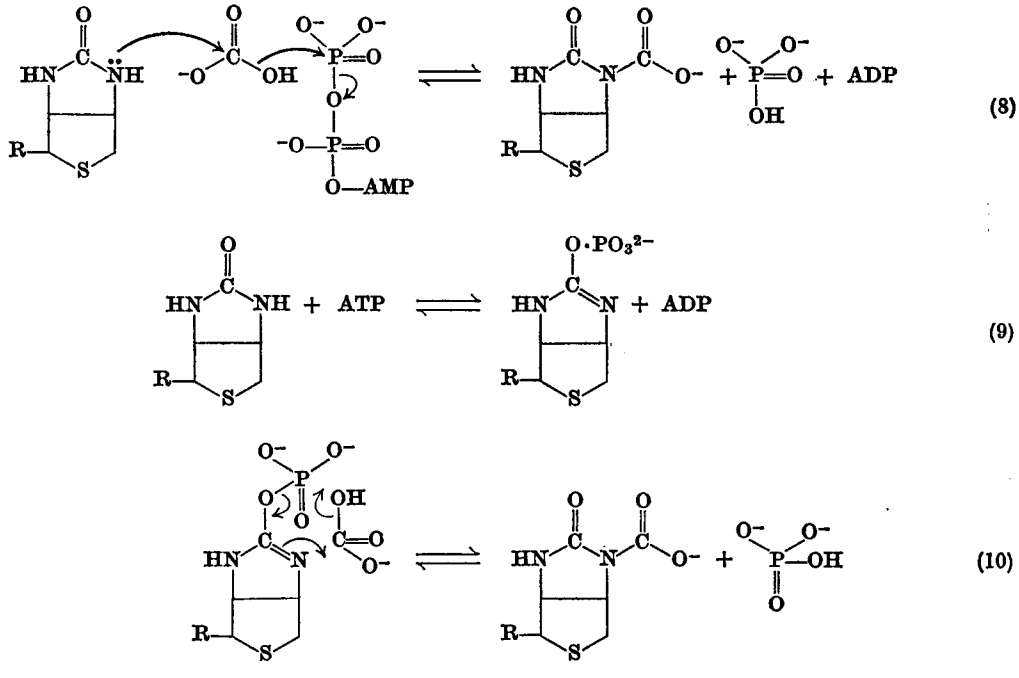
may also be expressed in thermodynamic terms. Wood *et al.* (1963*b*) determined the free energy of cleavage of the carboxybiotin enzyme according to eqn. (7):

$$H^+ + \text{Enzyme}\cdot\text{biotin}\cdot CO_2^- \rightleftharpoons \text{Enzyme}\cdot\text{biotin} + CO_2 \quad (7)$$

The observed value, $\Delta F^{0\prime} = -4{\cdot}7$ kcal./mole at pH 7·0, is sufficient to allow the compound to act as a carboxylating agent with suitable acceptors.

The exergonic nature of cleavage of the carboxybiotin enzyme also explains the ATP requirement of its formation from bicarbonate and the biotin enzyme. The chemical mechanism of this reaction is still under discussion. It is almost certain that bicarbonate rather than free carbon dioxide participates in the carboxylase reactions (Lynen *et al.* 1961; Kaziro, Hass, Boyer & Ochoa, 1962). However, the question whether the formation of the carboxybiotin enzyme is a concerted reaction, according to eqn. (8) (Scheme 5), or has to be subdivided into two steps, according to eqns. (9) and (10) (Scheme 5), with the intermediate formation of a phosphorylated biotin enzyme, has been raised. In view of the experiments of Scrutton & Utter (1965*b*), in which an ADP–ATP exchange was found to be catalysed by purified pyruvate carboxylase from avian liver, the second alternative must further be considered. Experiments with [^{18}O]bicarbonate and propionyl-CoA carboxylase do not allow a decision between the two alternative reaction mechanisms. In both cases the ^{18}O from bicarbonate will be recovered partly in the newly formed carboxyl group and partly in the liberated orthophosphate, as was found by Kaziro *et al.* (1962).

In discussing the function of carboxybiotin enzymes in carboxylation reactions it is worth noting that free carboxybiotin is significantly more stable than carboxybiotin enzyme. At pH 6·8 and 20° the half-life of free carboxybiotin was found to be 140 min. (Lynen *et al.* 1961; Knappe & Lynen, 1963), whereas that of carboxybiotin enzyme in the absence of substrate was 10 min. (Wood *et al.* 1963*b*). In addition, the carboxybiotin enzymes were found to be even more unstable in the presence of their substrates (Kaziro & Ochoa, 1961; Knappe *et al.* 1963; Knappe & Lynen, 1963). A change in conformation of the enzyme protein by binding with substrate may be responsible for this effect. If we take the spontaneous decarboxylation rate as a measure of transcarboxylation ability, it would appear that the protein moiety contributes considerably to the reactivity of the carboxyl group. This might involve hydrogen-bonding by a neighbouring group in the active centre to the 1′-N atom liberated during the transcarboxylation (Knappe & Lynen, 1963). Accordingly insight into



Scheme 5. Suggested mechanisms for the ATP-dependent carboxylation of biotin enzyme.

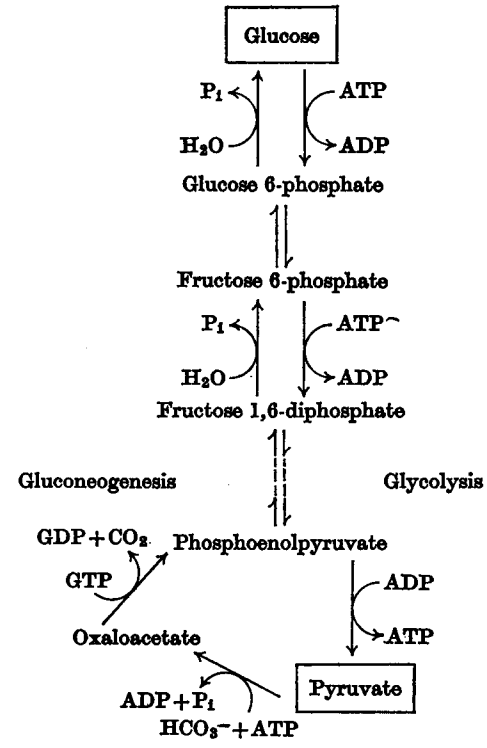
the three-dimensional structure at the active centre deserves special interest. In this respect our present knowledge is practically nil.

The situation might be more complicated by the fact that the biotin enzymes consist of aggregates. All the biotin enzymes that have been purified have been found to be of high molecular weight, approx. 700000, with about 4moles of bound biotin/mole of enzyme (Kaziro, Ochoa, Warner & Chen, 1961; Wood, Allen, Stjernholm & Jacobson 1963*a*; Himes *et al.* 1963; Scrutton & Utter, 1965*a*,*b*). Therefore it seems likely that these enzymes consist of four sub-units of molecular weight of 175000, each containing one molecule of biotin. This concept is supported by experiments of Kaziro & Ochoa (1964) with crystalline propionyl-CoA carboxylase. On ultracentrifugation in 7M-urea, the enzyme sediments as a single sharp peak with a sedimentation efficient of 2·55s instead of 19·75s for the native coenzyme. This suggests dissociation of the molecule into a number of sub-units of equal size, the molecular weight of which has not yet been determined. However, this treatment leads to irreversible inactivation of the enzyme.

The role of carboxylation in gluconeogenesis

The discovery of the biotin-dependent carboxylation reactions has greatly contributed to our understanding of the process of gluconeogenesis. Originally it was assumed that this process, consisting in the conversion of certain amino acids or of lactic acid into glucose, occurs via pyruvic acid and from there by the direct reversal of glycolysis. Doubt was thrown on this concept, however, when it was realized and especially emphasized by Krebs (1954) that three steps in the glycolytic sequence of reactions are not readily reversible (Scheme 6).

Two of the reactions in question are the two phosphorylations with ATP that initiate glycolysis by transforming glucose into fructose 1,6-diphosphate and are interlinked by the freely reversible isomerization of glucose 6-phosphate into fructose 6-phosphate. The third reaction is the generation of pyruvate by phosphate transfer from phosphoenolpyruvate to ADP. In the following years it was recognized that in gluconeogenesis these reactions are by-passed by other enzymes (for reviews see Krebs, Newsholme, Speake, Gascoyne & Lund, 1964; Weber, Singhal & Srivastava, 1965). The hydrolytic enzymes glucose 6-phosphatase and fructose diphosphatase replace the glycolytic enzymes glucokinase and phosphofructokinase. The most complicated of these circumventing steps is the sequence of reactions by which phosphoenolpyruvate is formed from pyruvate. This process involves several different enzymes and cofactors, and some of the sequential reactions occur in



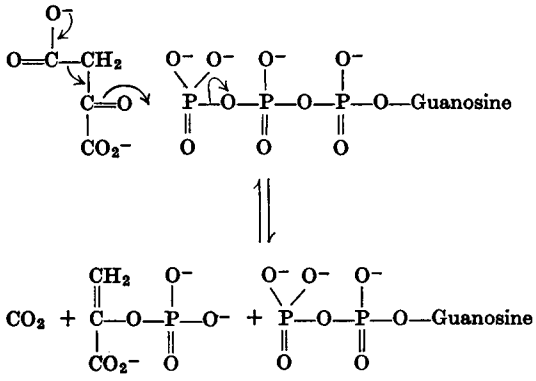
Scheme 6. Reaction paths of glycolysis and gluconeogenesis.

different compartments of the cell, necessitating the diffusion of intermediates from one compartment to another (Lardy, Paetkau & Walter, 1965*b*). In the centre of this by-pass function two enzymes that were discovered by Utter & Kurahashi (1954) and Utter, Keech & Scrutton (1964). One of the enzymes is the biotin-dependent pyruvate carboxylase, already mentioned, which synthesizes oxaloacetate from pyruvate and bicarbonate, and requires ATP. The oxaloacetate thus formed reacts then with GTP to form phosphoenolpyruvate, carbon dioxide and GDP (Scheme 6). This reaction is catalysed by phosphoenolpyruvate carboxykinase (Utter, 1961). Comparison of the overall balances of this detour with the pyruvate-kinase reaction reveals the great advantage of the former process with respect to phosphoenolpyruvate formation:

$$\text{Pyruvate}+\text{ATP} \rightleftharpoons \text{phosphoenolpyruvate}+\text{ADP} \quad (11)$$

$$\text{Pyruvate}+\text{ATP}+\text{GTP} \xrightleftharpoons{(CO_2)} \text{phosphoenolpyruvate}+\text{ADP}+\text{GDP}+P_i \quad (12)$$

In the process catalysed by pyruvate kinase (eqn. 11) only one 'energy-rich' pyrophosphate bond would be available, which cannot satisfy the



Scheme 7. Hypothetical mechanism of the carboxykinase reaction.

energetic requirement under physiological conditions. In the by-pass reactions, however (eqn. 12), two pyrophosphate bonds are consumed. This is achieved through first binding pyruvate to carbon dioxide at the expense of one phosphate bond. The bound pyruvate is then transferred to the terminal phosphate of GTP with release of carbon dioxide and the cleavage of the pyrophosphate bond between the β- and γ-phosphate groups of GTP. With regard to the chemical mechanism of the carboxykinase reaction, tracer experiments by Graves, Vennesland, Utter & Pennington (1956) have shown that the keto and not an enol form of oxaloacetate is the reactant. The concerted reaction, as shown in Scheme 7, would be compatible with this observation.

To emphasize the great advantage that is gained by the organisms if they use oxaloacetate, i.e. carboxylated pyruvate, instead of free pyruvate for the generation of phosphoenolpyruvate, the equilibrium constants of the kinase reaction and the carboxykinase reaction, as determined by Kurahashi, Pennington & Utter (1957) and McQuate & Utter (1959), are given:

Kinase reaction:

$$K_{eq.} = \frac{[\text{Phosphoenolpyruvate}][\text{ADP}]}{[\text{Pyruvate}][\text{ATP}]} = 1{\cdot}55 \times 10^{-4}\ (30°;\ \text{pH}\,7{\cdot}4)$$

Carboxykinase reaction:

$$K_{eq.} = \frac{[\text{Phosphoenolpyruvate}][\text{GDP}][\text{CO}_2]}{[\text{Oxaloacetate}][\text{GTP}]} = 0{\cdot}37\text{M}\ (30°)$$

An interesting feature of the pyruvate carboxylase isolated from rat or chicken liver is its absolute requirement for a catalytic amount of acetyl-CoA or other acyl-CoA compounds (Utter & Keech, 1960; Keech & Utter, 1963). It was therefore suggested that the acyl-CoA may serve as a control factor in the synthesis of oxaloacetate and glucose (Utter *et al.* 1964). Some support for this concept has come from the investigations by Krebs *et al.* (1964), who have shown that the rate of synthesis of glucose from lactate by kidney slices is influenced markedly by the addition of precursors of the acyl-CoA compounds. On the other hand, numerous studies in several Laboratories have revealed that the regulatory mechanisms in gluconeogenesis are rather complex and involve, besides pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose diphosphatase and glucose 6-phosphatase, i.e. all the so-called 'key gluconeogenic enzymes' (see Weber *et al.* 1965). The regulatory mechanisms function, not only by activation of inactive enzymes, but also by saturation of pre-existing enzymes or by changing the biosynthetic rate of enzymes (Weber, Singhal, Stamm, Fisher & Mentendiek, 1964; Lardy, Foster, Young, Shrago & Ray, 1965*a*; Henning, Seiffert & Seubert, 1963; Weber *et al.* 1965).

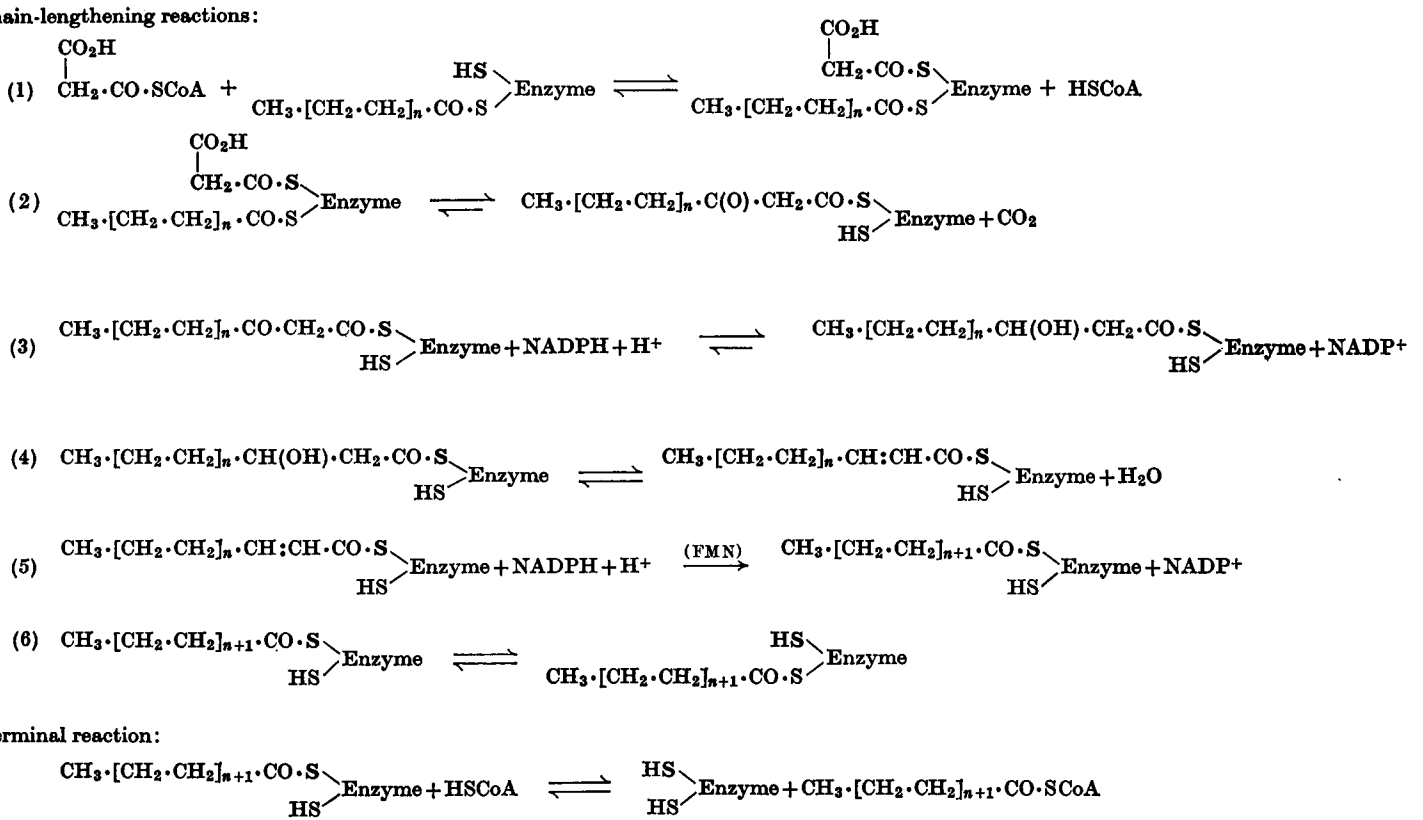
In addition the 'compartmentation' of enzymes, substrates and cofactors seems to play an important role in gluconeogenesis, at least in some animal species. Contrary to the situation in avian liver, where pyruvate carboxylase and phosphoenolpyruvate carboxykinase are both mitochondrial enzymes and therefore in close proximity, in rat liver the carboxykinase is located in the cytoplasm (Lardy *et al.* 1965*b*; Shrago & Lardy, 1966). Oxaloacetate formed in the mitochondria by the action of pyruvate carboxylase does not diffuse out, as such, into the cytoplasm. Instead it is reduced to malate or transaminated to aspartate, and in these forms transported from the mitochondria to the cytoplasm for gluconeogenesis. Shrago & Lardy (1966) demonstrated that the necessary enzymes exist in the cytoplasm for the reconversion of these substrates into oxaloacetate and hence into phosphoenolpyruvate. In addition, the rates of formation of phosphoenolpyruvate from malate and aspartate were found to be almost as great as those from oxaloacetate directly.

The role of carboxylation in fatty acid synthesis

The use of the energy of decarboxylation to circumvent a reaction that is not readily reversible was also found in studies on fatty acid synthesis. The first hint that biotin is in some way connected with fatty acid synthesis stems from experiments on the sparing effect of oleic acid and other unsaturated fatty acids on the biotin requirement of the lactic acid bacteria (Williams & Fieger, 1946; Williams, Broquist & Snell, 1947; Axelrod,

Priming reaction:

Chain-lengthening reactions:



Scheme 8. Mechanism of fatty acid synthesis.

Hofmann & Daubert, 1947). In fact, when oleate was added together with aspartate, there was no requirement for biotin in the growth medium. The first conclusive demonstrations of the participation of bicarbonate in the synthesis of long-chain fatty acids from acetate came from the work of Klein (1957) and of Gibson, Titchener & Wakil (1958). Shortly thereafter, Wakil, Titchener & Gibson (1958) showed that a biotin enzyme was involved in the process. This led to the hypothesis that malonyl-CoA might be an intermediate of fatty acid synthesis (see Lynen, 1959), which was proved within a short time by Brady (1958), by Wakil (1958) and in my Laboratory (Lynen, 1959). The biotin enzyme first purified by Wakil (1958) from avian liver turned out to be an acetyl-CoA carboxylase, which forms malonyl-CoA according to the following equation:

$$CH_3 \cdot CO \cdot SCoA + ATP + HCO_3^- \rightleftharpoons {}^-O_2C \cdot CH_2 \cdot CO \cdot SCoA + ADP + P_i \quad (13)$$

Insight into the chemical details of fatty acid synthesis, starting with malonyl-CoA, was first obtained in studies with the enzyme system of yeast (Lynen, 1960, 1961). From yeast cells, ruptured by vigorous shaking with glass beads, we were able to isolate a protein fraction 150 times as active as the crude extract. The purified enzyme, which we named fatty acid synthetase, proved to be homogeneous in the Tiselius apparatus and in the ultracentrifuge. Its molecular weight was estimated to be 2300000. It was found that 1 mole of freshly prepared fatty acid synthetase at 25° and pH 6·8 incorporates between 3200 and 5800 moles of malonyl-CoA into fatty acids/min.

The synthesis of fatty acids from malonyl-CoA requires NADPH as a reducing agent and small amounts of acetyl-CoA, in analogy with the avian and mammalian enzymes first studied by Wakil & Ganguly (1959) and Brady (1958). In contrast with these enzymes, however, the yeast enzyme does not synthesize primarily free palmitic acid but a mixture of palmitoyl-CoA and stearoyl-CoA (Lynen, Hopper-Kessel & Eggerer, 1964), according to eqn. (14) ($n = 7$–8):

$$\text{Acetyl-CoA} + n \text{ malonyl-CoA} + 2n \text{ NADPH} + 2n\, H^+ \rightarrow CH_3 \cdot [CH_2 \cdot CH_2]_n \cdot CO \cdot CoA + n\, CO_2 + n \text{ CoA} + 2n \text{ NADP}^+ + n\, H_2O \quad (14)$$

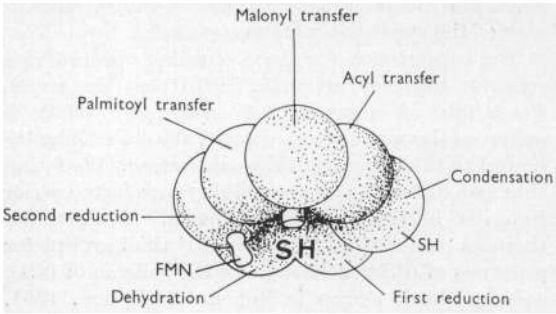
Acetyl-CoA serves as 'primer' of the process. Its C_2 unit is recovered only in the methyl end of the fatty acid produced (Lynen *et al.* 1964), indicating that C_2 units from malonyl-CoA are added to the acetyl residue during the synthetic reaction. In its function as 'primer', acetyl-CoA can be replaced by homologous saturated acyl-CoA compounds, but not by their oxidation products, identified in studies on fatty acid oxidation. Further, all attempts to find low-molecular-weight intermediates of the synthesis were unsuccessful.

The explanation for these puzzling observations was our discovery (Lynen, 1960) that the transformation of malonyl-CoA into fatty acids is achieved through intermediates that are covalently bound to thiol groups of the synthetase. We found that two different types of thiol groups have carrier function in the synthetic process. We denoted them as 'central' and 'peripheral' thiol groups for purposes of differentiation. In the scheme of fatty acid synthesis shown in Scheme 8 (Lynen, 1961, 1962), they are distinguished by **bold** and normal print.

The synthetic process is initiated by the transfer of an acetyl residue from acetyl-CoA to the 'peripheral' thiol group, designated as 'priming reaction'. It is followed by the transfer of a malonyl residue from malonyl-CoA to the 'central' thiol group. The next step is a condensation between the enzyme-bound acetyl and malonyl groups, resulting in the formation of acetoacetyl-enzyme with the concomitant liberation of carbon dioxide. The stepwise conversion of the β-oxo acid into the saturated acid is accomplished by way of its reduction by NADPH to D(−)-β-hydroxybutyryl-enzyme, followed by dehydration to crotonyl-enzyme and another NADPH-linked reduction to form the saturated butyryl-enzyme. In the second reduction step FMN serves as hydrogen carrier. All the acyl residues involved in these chemical transformations are bound to the 'central' thiol group. At the stage of the saturated acid finally the butyryl group is transferred to the 'peripheral' thiol group, thus liberating the 'central' thiol group for introduction of the next malonyl residue. The reaction cycle can then proceed again, starting with butyryl-malonyl-enzyme, and is repeated until long-chain (C_{16} or C_{18}) saturated fatty acids are formed. In the terminal reaction step the acyl residue of palmitoyl- or stearoyl-enzyme is transferred from the 'central' thiol group to CoA with the formation of palmitoyl-CoA or stearoyl-CoA and the regenerated enzyme. The free enzyme can again react with acetyl-CoA and malonyl-CoA, thereby reinitiating the entire process.

This whole sequence of reactions is accomplished by a multienzyme complex. Its functional unit was proposed to consist of a combination of seven different enzymes arranged around the 'central' thiol group in such a manner that the intermediates bound covalently to this group can come in close contact with the active sites of the participating enzymes (Scheme 9).

Structural details of the multienzyme complex from yeast are largely hypothetical. For example, it is still uncertain whether the 'central' thiol group



Scheme 9. Hypothetical structure of the multienzyme complex of fatty acid synthetase. The seven enzyme units shown refer to the seven reactions of Scheme 8.

belongs to an individual structural element without enzymic activity, as we assumed in our model, or whether this group is covalently bound to one of the enzymic components. We prefer the first alternative, since the architecture of the fatty acid synthetase from yeast would resemble the structure of the analogous enzyme system from bacteria. As the elegant experiments of Majerus, Alberts & Vagelos (1965*a*) with the *Escherichia coli* system have demonstrated, the 'central' thiol group, which carries the fatty acid intermediates, is bound to a readily dissociable protein of molecular weight about 9500, designated 'acyl carrier protein'. The bacterial enzyme system is not arranged in a stable multienzyme complex. By using standard methods of protein fractionation, it was possible to separate this enzyme system into enzymically active individual components (Majerus, Alberts & Vagelos, 1964; Wakil, Pugh & Sauer, 1964). A similar 'acyl carrier protein' has been reported to be present in the corresponding enzyme system from plants (Overath & Stumpf, 1964).

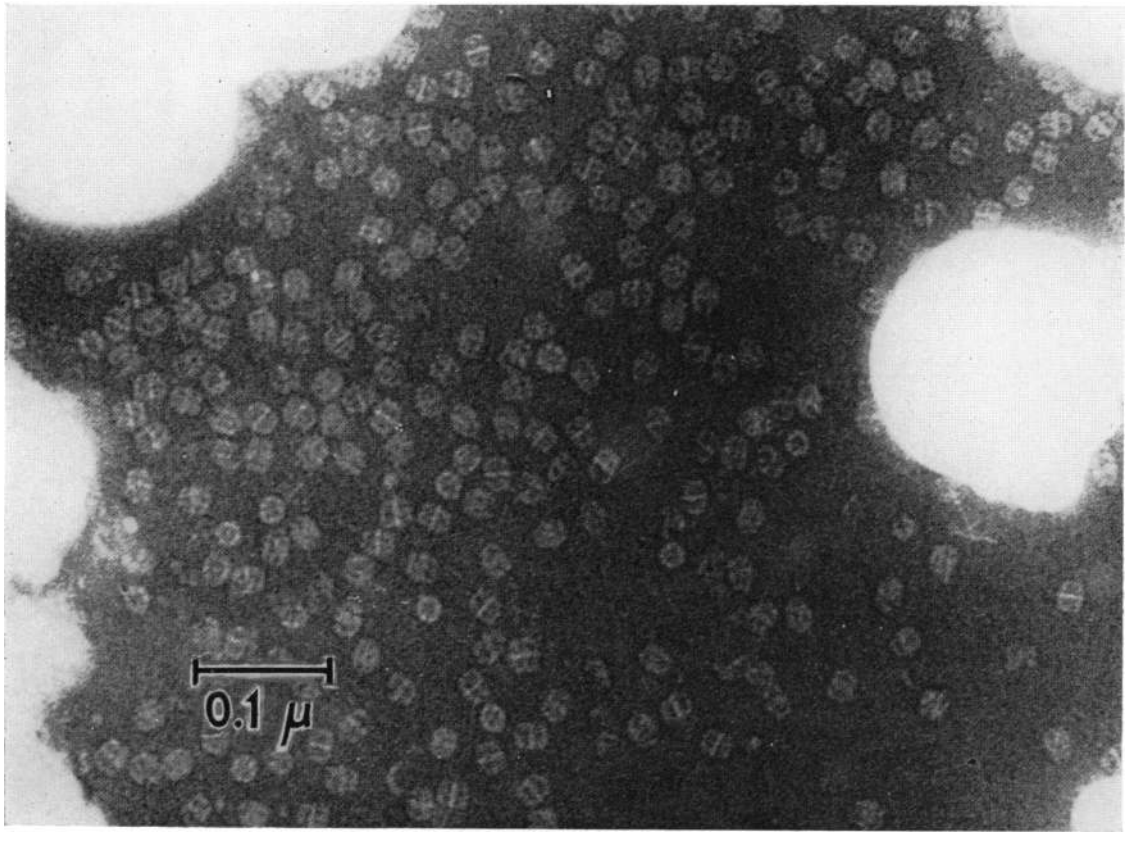
Our repeated attempts to split the multienzyme complex of yeast into its sub-units with retention of the individual enzyme activities were without success. To split the complex, it was necessary to use such drastic conditions, e.g. 0·2M-sodium deoxycholate or 6M-urea, that most of the individual enzyme activities of fatty acid synthesis were lost (Hagen, 1963). With respect to its stability the multienzyme complex of yeast fatty acid synthetase resembles the avian and mammalian synthetase complexes (Bressler & Wakil, 1961; Larrabee, McDaniel, Bakerman & Vagelos, 1965; Brady, 1960; Hsu, Wasson & Porter, 1965).

From the standpoint of chemical mechanisms all fatty acid synthetases studied so far seem to catalyse the same reaction sequence (Lynen, 1961, 1962; Majerus *et al.* 1964; Alberts, Majerus, Talamo & Vagelos, 1964; Wakil *et al.* 1964; Sauer, Pugh, Wakil, Delaney & Hill, 1964; Hsu *et al.* 1965). One minor dissimilarity concerns the terminal reaction, which yields palmitoyl-CoA and stearoyl-CoA with the yeast synthetase but free palmitate with the animal and bacterial enzyme systems. This difference may be due to the intervention of a hydrolytic enzyme that replaces the acyltransferase in the terminal reaction.

The structural organization of the fatty acid synthetase from yeast was verified by electron microscopy (Plate 1). By use of the negative staining technique with phosphotungstic acid Hagen & Hofschneider (1964) were able to recognize single particles of oval shape surrounded by an equatorial ring. The longitudinal diameter of the particles is 250Å, their cross diameter 210Å. Unfortunately we cannot yet translate the information from electron micrographs into known schemes of structure. However, a structure composed of three circular sub-units fitting together seems possible. From the results of the chemical studies we have good evidence that supports the concept that each particle of molecular weight 2300000 is composed of three functional assemblies.

To demonstrate the manifold catalytic activities attributed to the synthetase, we used model substrates in which the carboxylic acid intermediates of fatty acid synthesis were bound to pantetheine or *N*-acetylcysteamine (Lynen, 1961, 1962) (Scheme 10). Lacking the strong covalent bond to the 'central' thiol group of the natural substrates, the affinity of these model substrates for the component enzymes is rather small. This defect can be circumvented, however, by employing high concentrations of the model substrates. The differences in affinity are schematically illustrated for the first reductive reaction in Scheme 11. In studies of the bacterial enzyme system, the natural substrates, i.e. the carboxylic acid intermediates bound to the 'acyl carrier protein', could be used (Majerus *et al.* 1964; Alberts *et al.* 1964; Wakil *et al.* 1964; Sauer *et al.* 1964).

In addition to the experiments with model substrates we also used stoicheiometric amounts of the yeast enzyme and demonstrated the enzyme-bound intermediates directly (Lynen, 1962). As an example, short incubation of [1-^{14}C]acetyl-CoA with synthetase led to the formation of the radioactive acetyl-enzyme (Scheme 12). It could be precipitated with trichloroacetic acid with retention of radioactivity, indicating that the labelled acetyl group was covalently linked to the protein. When the radioactive acetyl-enzyme was separated from excess of substrate by passing the reaction mixture over Sephadex and then incubated with malonyl-CoA and NADH, more than 95% of the protein-bound acetic acid could be recovered in the fatty acids formed (E. Schweizer, unpublished work).

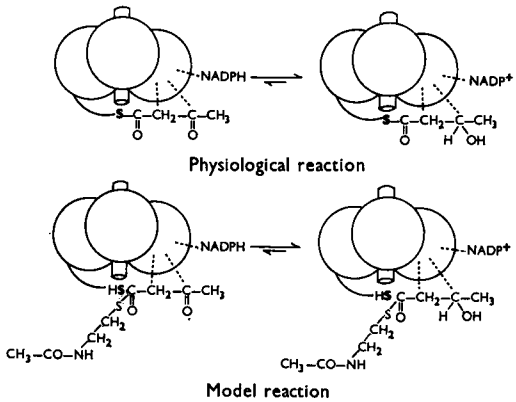


EXPLANATION OF PLATE 1

Electron micrograph of the purified fatty acid synthetase from yeast.

Malonyl transfer: [^{14}C]Malonyl-CoA + pantetheine $\rightleftharpoons$ CoA + [^{14}C]malonylpantetheine
Palmitoyl transfer: Palmitoyl-CoA + [^{14}C]CoA $\rightleftharpoons$ CoA + palmitoyl-[^{14}C]CoA
First reduction: Acetoacetyl-S-AC + NADPH + H^+ $\rightleftharpoons$ D(−)-β-hydroxybutyryl-S-AC + $NADP^+$
Second reduction: Crotonyl-S-AC + NADPH + H^+ $\xrightarrow{(FMN)}$ butyryl-S-AC + $NADP^+$
Dehydration: D(−)-β-Hydroxybutyryl-S-AC $\rightleftharpoons$ crotonyl-S-AC + H_2O
Condensation: Acetoacetyl-S-AC + $^{14}CO_2$ + HSCoA $\rightleftharpoons$ [^{14}C]malonyl-S-AC + acetyl-SCoA

Scheme 10. Summary of assay reactions with model substrates for the determination of the enzymic components of fatty acid synthetase. -S-AC, $-S\cdot CH_2\cdot CH_2\cdot NH\cdot CO\cdot CH_3$.



Scheme 11. Schematic picture of the interaction of the model substrate with a component of the multienzyme complex that catalyses the first reduction.

If the incubation mixture of [1-^{14}C]acetyl-CoA and synthetase was supplemented with malonyl-CoA a radioactive acetoacetyl-enzyme was formed (see Scheme 8). This could also be precipitated with trichloroacetic acid and yielded [3-^{14}C]acetoacetate after mild alkaline hydrolysis (Lynen, 1962, 1964). Our attempts to isolate the native acetoacetyl-enzyme by employing the Sephadex technique failed, because acetoacetyl-enzyme is slowly decomposed spontaneously to free acetoacetate. This is illustrated by the experiment of Fig. 1, where the kinetics of the formation of free and bound acetoacetyl residues was estimated. During the first 2min. protein-bound acetoacetate, i.e. acetoacetyl-enzyme, was formed in a very fast reaction. Subsequently free acetoacetate was produced at a much lower rate, representing the slow hydrolysis of acetoacetyl-enzyme, which was continually regenerated by the interaction of excess of acetyl-CoA and malonyl-CoA with the liberated enzyme. The quantity of acetoacetate bound to the enzyme agrees well with the concept that the fatty acid-synthetase complex is a trimer (D. Oesterhelt, unpublished work).

We also measured the equilibrium constant of the formation of acetoacetyl-enzyme according to eqn. (15):

$$\text{Acetyl-CoA} + \text{malonyl-CoA} + \text{enzyme} + H^+ \rightleftharpoons \text{acetoacetyl-enzyme} + 2\,\text{CoA} + CO_2 \quad (15)$$

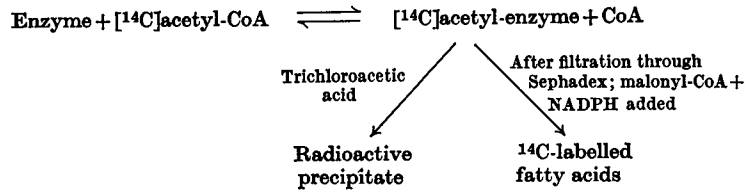
$$\text{Acetyl-CoA} + \text{acetyl-CoA} \rightleftharpoons \text{acetoacetyl-CoA} + \text{CoA} \quad (16)$$

and found the value (Lynen, 1965):

$$K_{eq.} = \frac{[\text{Acetoacetyl-enzyme}][\text{CoA}]^2[CO_2]}{[\text{Acetyl-CoA}][\text{malonyl-CoA}][\text{enzyme}][H^+]} = 2\times10^5\ (0°)$$

Eliminating the H^+ concentration in this equation the equilibrium constant at pH 7·0 equals:

$$K'_{eq.} = \frac{[\text{Acetoacetyl-enzyme}][\text{CoA}]^2[CO_2]}{[\text{Acetyl-CoA}][\text{malonyl-CoA}][\text{enzyme}]} = 2\times10^{-2}\text{M}$$



Scheme 12. Scheme of the formation and transformation of [14C]acetyl-enzyme.

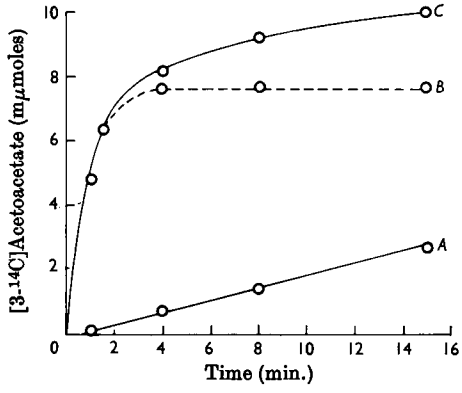


Fig. 1. Kinetics of the formation of acetoacetyl-enzyme and free acetoacetate. Each of the samples contained in a volume of 2·0 ml.: potassium phosphate, pH 6·5, 150 μmoles; cysteine, 20 μmoles; malonyl-CoA, 0·1 μmole; [1-14C]-acetyl-CoA ($1·33 \times 10^7$ counts/min./μmole), 0·05 μmole; yeast synthetase (specific activity 1 enzyme unit/mg.), 4·6 mg. The mixtures were incubated at 0°. At the times indicated protein-bound and free [3-14C]acetoacetate were measured as described by Lynen *et al.* (1964): *A*, free [14C]acetoacetate; *B*, protein-bound [14C]acetoacetate; *C*, free + protein-bound [14C]acetoacetate.

If we compare this equilibrium constant (condensation with malonyl-CoA) with the equilibrium constant of the thiolase reaction according to eqn. (16) (condensation with acetyl-CoA), which also generates an acetoacetyl thio ester, the great thermodynamic advantage gained by the decarboxylation accompanying the condensation with malonyl-CoA becomes evident. The equilibrium constant of the thiolase reaction was found (Lynen & Decker, 1957) to be:

$$K_{eq.} = \frac{[\text{Acetoacetyl-CoA}][\text{CoA}]}{[\text{Acetyl-CoA}]^2} = 1·6 \times 10^{-5}\ (25°;\ \text{pH}\ 7·0)$$

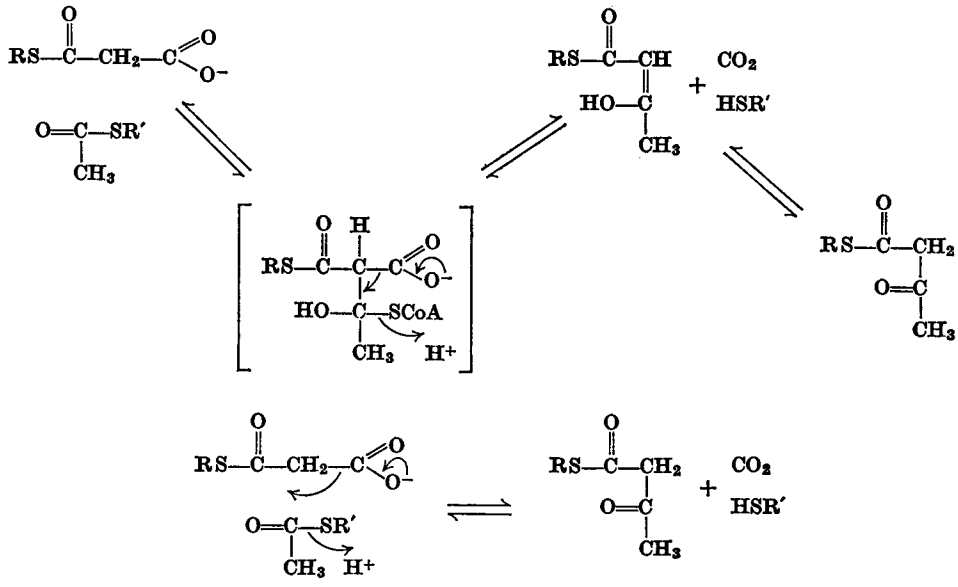
This great advantage with respect to the synthesis of carbon chains is utilized in Nature not only in the process of fatty acid biosynthesis but also in the biosynthesis of many other natural products (Lynen & Tada, 1961)—including aromatic ring compounds, tetracyclines, various macrolides and acetylene derivatives—which all conform to the polyacetate rule of Birch (1957).

The favourable shift in the equilibrium position of the condensation reaction is ultimately due to the delivery of energy by ATP. At the expense of one pyrophosphate bond acetyl-CoA is first bound to carbon dioxide as a carrier (eqn. 13). After replacement of CoA by the 'central' thiol group of the enzyme complex condensation occurs with the acetyl residue bound to the 'peripheral' thiol group. The condensation is accompanied by the release of carbon dioxide and the cleavage of one thio ester bond. Because both reactants are in an 'activated' state, the condensation reaction becomes very efficient.

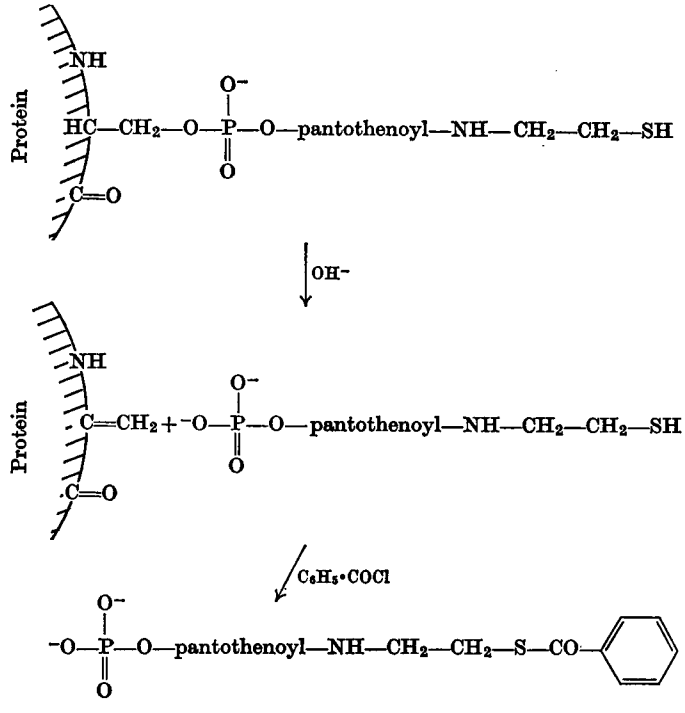
From a chemical standpoint the condensation reaction may be classified as an acylation of malonic ester. The methylene group of the malonyl thio ester, which is known to be more nucleophilic than the methyl group of an acetyl thio ester, adds to the electrophilic carbonyl carbon of the sulphur-bound carboxylic acid, as shown in the upper half of Scheme 13. The intermediate formed is converted into the β-oxoacyl derivative by the subsequent elimination of thiol and carbon dioxide. An alternative reaction mechanism might be the concerted process shown in the lower half of Scheme 13. A decision between the two mechanisms may be reached by tracer experiments with heavy water.

Investigation of the chemical nature of the two types of thiol groups involved in fatty acid synthesis first led to the identification of the 'peripheral' one as belonging to cysteine (Lynen, 1964). It was found that 1 mole of yeast synthetase contains 3 moles of 'peripheral' thiol groups, which supports again the concept of a trimer. We have obtained experimental evidence that the 'peripheral' thiol groups are located in the condensing-enzyme components of the multienzyme complex.

The carrier of the 'central' thiol group of the yeast fatty acid synthetase was recently identified as 4′-phosphopantetheine probably bound through phosphodiester linkage with the hydroxyl group of a serine residue of the polypeptide (E. Schweizer,



Scheme 13. Mechanism of the formation of β-oxo acids from malonyl thio ester.

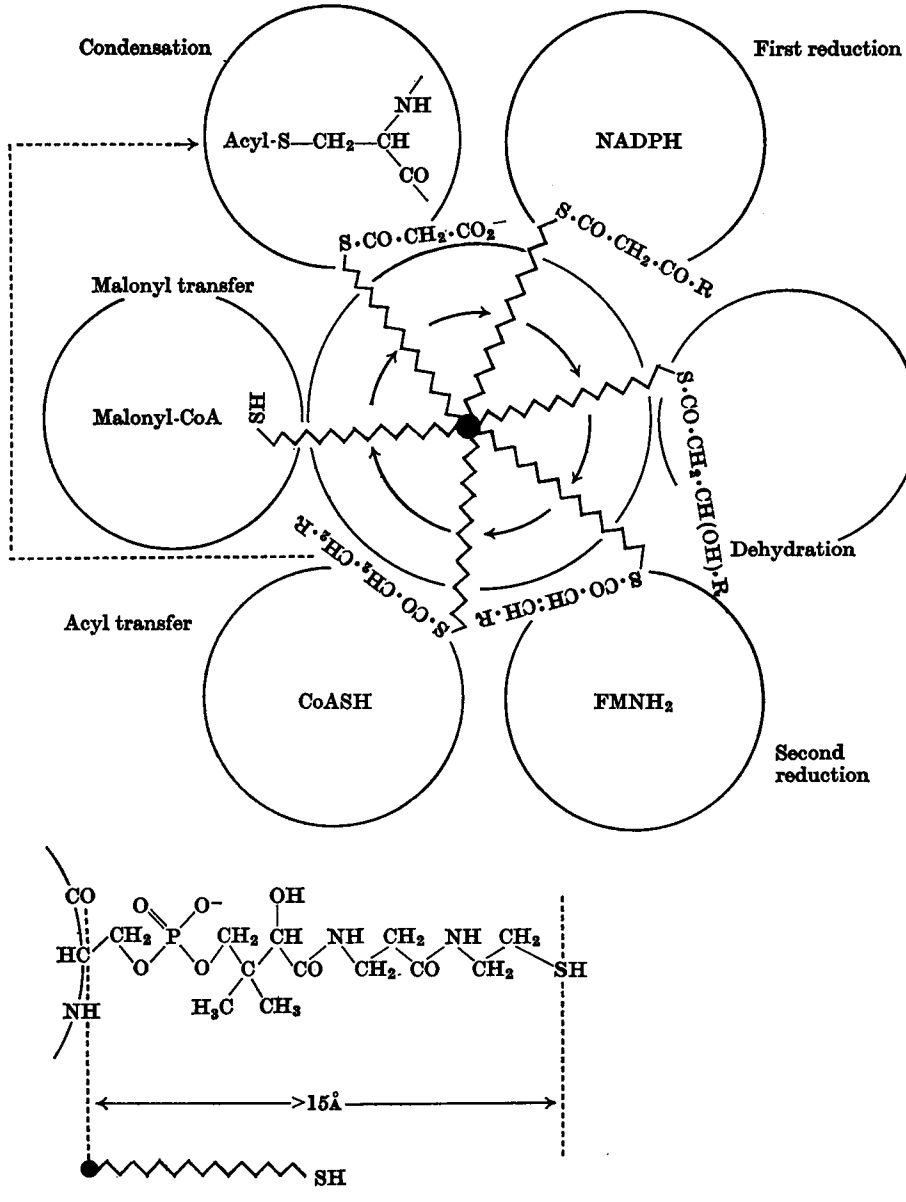


Scheme 14. Schematic representation of the identification of protein-bound 4′-phosphopantetheine.

unpublished work). This kind of attachment was discovered by Majerus, Alberts & Vagelos (1965*b*) and by Pugh & Wakil (1965) in studies on the chemical structure of the 'acyl carrier protein' of *E. coli*, and was also found in the mammalian fatty acid-synthetase complex (Larrabee *et al.* 1965).

Our evidence for the occurrence of 4′-phosphopantetheine stems from experiments in which the purified fatty acid synthetase of yeast was heated at pH 12 at 98° for 1 hr., following the procedure described by Majerus *et al.* (1965*b*). Under these conditions the protein released a low-molecular-weight compound, which after benzoylation and further purification was identified as *S*-benzoyl-4′-phosphopantetheine by chemical analysis and by comparison with the authentic compound (Scheme 14). The release of 4′-phosphopantetheine by mild alkaline treatment is in accord with the concept of an elimination reaction in the β-position of a polypeptide-bound serine.

The presence of 4′-phosphopantetheine is further supported by experiments with yeast cells grown in a medium containing ^{14}C-labelled pantothenic acid. Wells, Schultz & Lynen (1966) isolated the



Scheme 15. Reaction scheme illustrating individual events during fatty acid synthesis on the multienzyme complex.

fatty acid synthetase from these cells and found the purified enzyme to contain 3moles of radioactive pantothenate covalently bound/mole of enzyme.

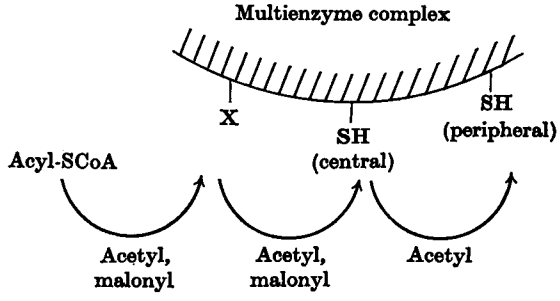
The attachment through 4′-phosphopantetheine seems to be very appropriate for the functioning of the multienzyme complex. It provides a flexible arm of more than 15Å length carrying the 'central' thiol group and conceivably permitting rotation of the latter between the various enzymes. In this manner it is easily possible to bring the fatty acid intermediates, bound covalently to this thiol group, in close contact with the active site of each component enzyme, which has only limited freedom of motion in the stable multienzyme complex. This is schematically illustrated in Scheme 15, where the circles should indicate the active sites of the participating enzymes.

During our investigations which led to the identification of the 'peripheral' and 'central' thiol groups we found to our great surprise that acetate and malonate are not bound to the enzyme complex exclusively via sulphur atoms. In these experiments we used the lability of thio esters towards performic acid as a tool for the characterization. Performic acid oxidizes thio esters to the corresponding sulphonic acids with release of the carboxylic acids (Harris, Meriwether & Harting-Park, 1963) (eqn. 17):

$$R{\cdot}S{\cdot}CO{\cdot}R' \xrightarrow{HCO{\cdot}O{\cdot}OH} R{\cdot}SO_3H + R'{\cdot}CO_2H \quad (17)$$

In Table 2 the results of an experiment with [^{14}C]acetyl-enzyme are shown. The labelled enzyme was prepared by the interaction of constant amounts of enzyme with various concentrations of [1-^{14}C]-acetyl-CoA, followed by precipitation with trichloroacetic acid and measuring the protein-bound radioactivity. Table 2 shows that the transfer of radioactive acetate to the enzyme depends on the concentration of acetyl-CoA used, as expected, if the acetyl transfer between acetyl-CoA and enzyme is a reversible process. However, in the whole concentration range studied, only about 50% of the radioactive acetyl groups bound to the protein were released by treatment with performic acid.

From this and other experiments we came to the conclusion that the acyl transfer to the multienzyme complex is initiated with the transfer to some non-thiol acceptor group X in the protein. As illustrated in Scheme 16 malonyl and acetyl residues are transferred from group X to the 'central' thiol group. The further transfer to the



Scheme 16. Specificity of acyl transfer to the different acceptor groups on the multienzyme complex.

Table 2. *Formation of* [^{14}C]*acetyl-enzyme and release of* [^{14}C]*acetic acid by oxidation with performic acid*

Each sample contained in 1·0ml. of 0·1M-potassium phosphate buffer, pH6·5: 2mg. of yeast fatty acid synthetase (specific activity 850milliunits/mg.), 10μmoles of cysteine and [^{14}C]acetyl-CoA (24·8$\times$10^6 counts/min./μmole) in the concentrations listed. The mixture was incubated for 5min. at 0° and the reaction stopped by the addition of 0·3ml. of 3N-trichloroacetic acid. After careful washing, the precipitated protein was dissolved in 0·5ml. of 98% formic acid, and 0·2ml. samples were dried on strips of Whatman no. 1 paper and the radioactivity was measured before and after 12hr. exposure to an atmosphere of performic acid. Before measurement in the scintillation counter the paper strips were dried *in vacuo* over KOH. The radioactivity values listed are based on 2mg. of protein.

Concn. of [^{14}C]acetyl-CoA (μM)	Protein-bound radioactivity (counts/min.) Direct	After oxidation	Stable radioactivity (%)
2	15623	7825	50
4	19582	9100	46·5
6	23847	10045	42
10	29325	13260	45
15	38590	17270	45
20	39830	17731	45
30	40817	18921	46
40	45437	20741	45·5
50	50113	22921	45·5

'peripheral' thiol group is specific for acetate and its higher homologues.

According to our scheme, the acyl residue of malonyl-enzyme is bound to both group X and the 'central' thiol group. In agreement with this assumption, three radioactive acyl-peptides were isolated from the peptic hydrolysate of [3-^{14}C]-malonyl-enzyme by chromatography on DEAE-Sephadex. This is illustrated in Fig. 2. The fastest-moving fraction, signified A, was split by performic acid, whereas fractions B and C were stable to the same treatment. From the acid hydrolysate of malonyl-peptide A, cysteamine and β-alanine were identified by ion-exchange chromatography (E. Schweizer, unpublished work). Since cysteine was absent, this peptide is derived from the polypeptide area around the 'central' thiol group.

The radioactivity of fractions B and C was due to the presence of a [^{14}C]malonyl-heptapeptide and a [^{14}C]malonyl-pentapeptide, which could be purified by chromatographic procedures. The acyl-pentapeptide contained serine besides histidine, glycine, alanine and leucine (*C*-terminal position). The acyl-heptapeptide was similar but contained, in addition, glycine and glutamic acid. These results seem to indicate that group X is identical with the hydroxyl group of serine.

At the present time we are still hesitant to accept this assumption. From our measurement of the equilibrium constant of the malonyl transfer between malonyl-CoA and the enzyme it follows that the bond energy of malonyl-enzyme is somewhat higher than that of malonyl-CoA (D. Oesterhelt, unpublished work). The bond energy of *O*-malonylserine, however, would be expected to be lower. Whether this argument also applies to an acyl-serine inserted into the complex structure of a protein remains to be elucidated. Under these circumstances the energetics could be quite different, especially if negatively charged groups are present at the binding site and repel the malonyl anion. On the other hand, one must consider also the possibility that the acyl residue in the native enzyme is bound to some very reactive group—possibly the imidazole ring of a histidine residue—and is only transferred to the serine hydroxyl group spontaneously during denaturation. In order to answer these questions further experiments are required. They should also indicate whether the carrier group X is connected with the enzyme components that catalyse acyl transfer reactions.

Another unsolved problem is why fatty acid synthetase produces mainly palmitic acid and stearic acid but no carboxylic acids of shorter chain length. Earlier we assumed that this might be due to the specificity of the enzymic component that terminates the process by transferring the fatty acid radical from the enzyme to CoA (Lynen, 1961). However, when Schweizer (1963) studied the relationship between rate of acyl transfer and chain length of the acid, it was found to our great surprise that the saturated acids from C_6 to C_{20} were transferred at nearly equal rates. Consequently other factors must be responsible for stopping the synthesis at the stage of the C_{16} and C_{18} acids.

During experiments on the protection of fatty acid synthetase against the inactivation by the

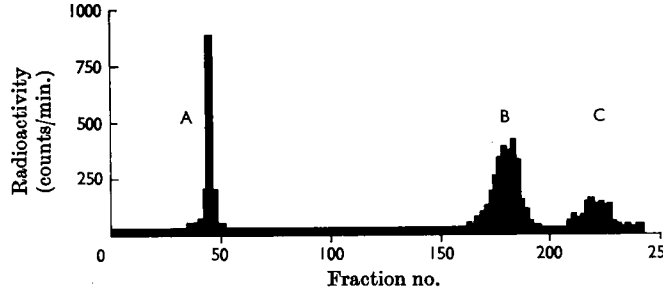


Fig. 2. Chromatography of the peptic hydrolysate of [^{14}C]malonyl-enzyme on DEAE-Sephadex. For the preparation of [^{14}C]malonyl-enzyme 1·6g. of synthetase (specific activity 550 milliunits/mg.) and 28μmoles of [3-^{14}C]malonyl-CoA (1·2$\times$10^6 counts/min./μmole) in 120ml. of 0·05M-potassium phosphate buffer, pH 6·5, were incubated for 2min. at 22°. The reaction was stopped by the addition of 6ml. of 3N-trichloroacetic acid. After careful washing, the precipitated protein was suspended in 2l. of 0·01N-HCl and digested by incubation with 160mg. of crystalline pepsin at room temperature. After evaporation of the water *in vacuo* the residue was dissolved in 7ml. of pyridine–acetate buffer, pH 6·25. Insoluble material was removed by centrifugation, and the clear solution chromatographed on a column (2·1cm.$\times$150cm.) of DEAE-Sephadex. The eluting solvent until fraction 70 (volume of fractions, 6ml.) was 0·05M-pyridine–acetate buffer, pH 6·25; thereafter a gradient was employed. For this purpose 0·2N-acetic acid was continuously introduced into a mixing chamber containing 200ml. of 0·05M-pyridine–acetate buffer, pH 6·25.

Table 3. *Protection of synthetase from inactivation with N-ethylmaleimide by preincubation with acyl-CoA derivatives*

Equal amounts of synthetase were preincubated with the CoA derivatives listed. *N*-Ethylmaleimide (final concn. 5mM) was added and the mixture was incubated for 5min. at 0°. Excess of inhibitor was then removed by the addition of cysteine, after which the enzyme activity was measured and compared with the activity of the untreated enzyme (=100).

Acyl-CoA during preincubation (0·28mM)	Relative enzyme activity
—	5·8
Malonyl-CoA	4·8
Acetyl-CoA	84·6
Butyryl-CoA	74·1
Decanoyl-CoA	66·9
Myristoyl-CoA	48·4
Palmitoyl-CoA	33·7
Untreated enzyme	(100)

thiol reagent *N*-ethylmaleimide (Lynen, 1962) an observation was made that may give a clue. As Table 3 illustrates, preincubation of the enzyme with acetyl-CoA or homologous saturated acyl-CoA derivatives, leading to the acylation of both the 'central' and 'peripheral' thiol groups, protected the synthetase against the action of *N*-ethylmaleimide. In contrast, however, preincubation of the synthetase with malonyl-CoA, which acylates only the 'central' thiol group, had no protective effect. We must conclude that the protection is due to the acylation of the 'peripheral' thiol group. Comparison of the effect of equal concentrations of the homologous saturated acyl-CoA derivatives (Table 3) shows that protection decreases with increasing chain length of the acyl residue. This would mean that acetyl radicals can acylate to a much greater extent than palmitoyl radicals. As an explanation one could speculate that the environment of the 'peripheral' thiol group of the multienzyme complex is hydrophilic, whereas that of the 'central' one is lipophilic. Thus with increasing chain lengths of the fatty acids the tendency to move from the 'central' to the 'peripheral' thiol group would gradually decrease. Palmitic acid and stearic acid would preferentially remain on the 'central' thiol group, thereby blocking the uptake of a new malonyl radical until the fatty acid is released from the enzyme complex by transfer to CoA. If this speculation is correct, it would mean that the architecture of the complex, which depends on highly specific protein–protein interactions between the component enzymes, carries specific information about the reaction product.

Table 4. *K_i values of various fatty acyl-CoA derivatives and K_m value of citrate*

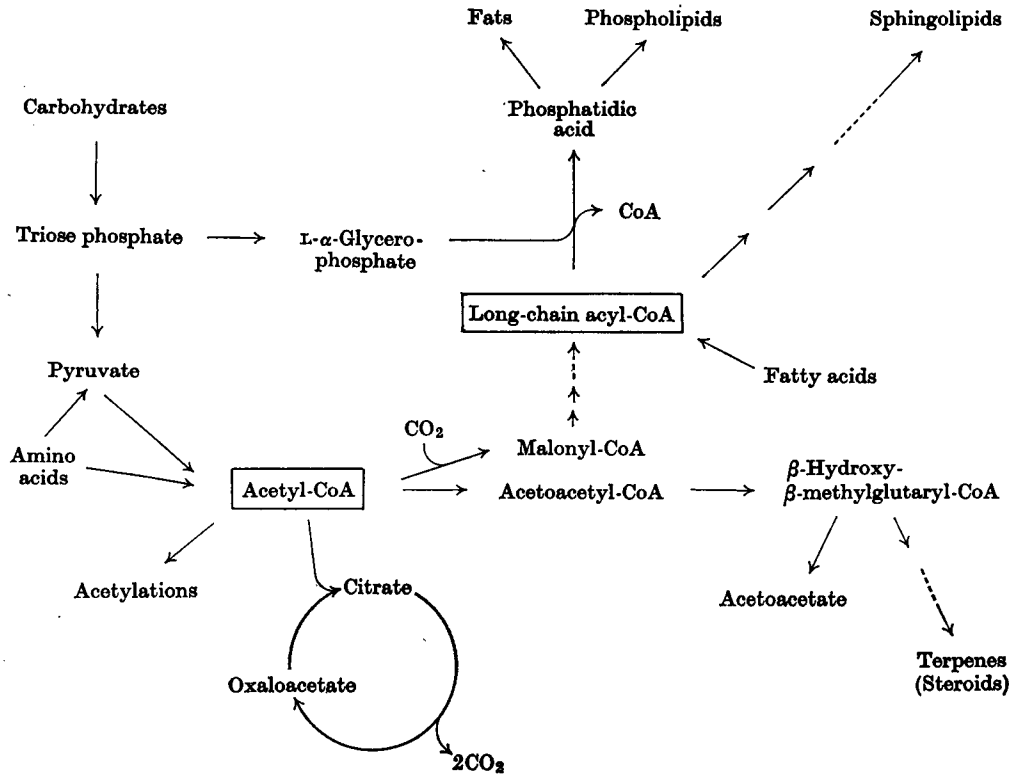
The results were obtained by Numa *et al.* (1965*b*).

Effector	K_i (M)
Stearoyl-CoA	$3·3\times10^{-7}$
Oleoyl-CoA	$6·7\times10^{-7}$
Margaroyl-CoA	$3·6\times10^{-7}$
Palmitoyl-CoA	$8·1\times10^{-7}$
Palmitoleoyl-CoA	$2·5\times10^{-6}$
Myristoyl-CoA	$2·6\times10^{-5}$
Decanoyl-CoA	$3·5\times10^{-5}$
	K_m (M)
Citrate	$3·9\times10^{-3}$

In conclusion I would like to discuss briefly our present views about the rate control of fatty acid synthesis (Numa, Bortz & Lynen, 1965*a*). This will bring us back to the biotin enzyme acetyl-CoA carboxylase, the step recognized to be rate-limiting in fatty acid synthesis in warm-blooded organisms (see Numa *et al.* 1965*a*). As has been shown in several Laboratories one of the unique features of acetyl-CoA carboxylase from animal sources is its activation by tri- and di-carboxylic acids, especially citrate (Martin & Vagelos, 1962; Waite & Wakil, 1963; Matsuhashi, Matsuhashi & Lynen, 1964). Extensive studies of this stimulatory effect have revealed that the increased activity is connected with an aggregation of the enzyme (Vagelos, Alberts & Martin, 1963; Numa, Ringelmann & Lynen, 1965*b*; Numa & Ringelmann, 1965). The citrate concentration required for half-maximal activation was found to be 3·9mM (Numa *et al.* 1965*b*). This observation appears to exclude the possibility of physiological control of fatty acid synthesis through the cellular concentration of citric acid.

Another interesting property of the acetyl-CoA carboxylase is its inhibition by long-chain acyl-CoA derivatives, discovered by Bortz & Lynen (1963*a*). Numa *et al.* (1965*b*) carried out systematic kinetic studies on the inhibition by palmitoyl-CoA with the purified enzyme, and found the inhibition to be competitive with regard to citrate, the enzyme activator. From experiments with various long-chain fatty acyl-CoA derivatives the K_i values listed in Table 4 were calculated and found to decrease with increasing chain length of the acyl radical. If one compares saturated acids and unsaturated acids of the same chain length, the unsaturated acids are less inhibitory.

Our studies have been restricted to the properties of the enzyme *in vitro* and must be cautiously interpreted with regard to the physiological regulation of fatty acid synthesis. It is, nevertheless, conceivable that some of our findings might



Scheme 17. Biological pathway of lipogenesis.

in fact represent part of the cellular control mechanism. Raised concentrations of fatty acids in the blood are associated with starvation and diabetes, conditions in which fatty acid synthesis is known to be almost fully blocked (Fritz, 1961). Further, it could be shown in normal animals that a diet rich in fat or the infusion of chylomicrons led to a drastic inhibition of fatty acid synthesis.

Related to this observation it was found that the concentration of the higher acyl-CoA compounds in liver is markedly increased under all conditions of depressed fatty acid synthesis (Bortz & Lynen, 1963*b*; Tubbs & Garland, 1964; Wieland, Weiss, Eger-Neufeldt, Teinzer & Westermann, 1965). Therefore it is tempting to speculate that fatty acid synthesis is under typical feedback control. As can be seen from the pattern of lipogenesis (Scheme 17), the inhibitory long-chain acyl-CoA derivatives represent the last molecules in the synthetic sequence before subsequent incorporation into the 'complex lipids'. Further, the inhibition would affect the enzyme acetyl-CoA carboxylase, the point at which fatty acid synthesis branches off from the many other reaction paths of acetyl-CoA.

In my lecture I have discussed mainly work carried out in our Laboratory within the last 8 years. I want to acknowledge the exceptional labours of William Chan, Christa Duba, Sabine Günther, Alexander Hagen, Richard Himes, Ingrid Hopper-Kessel, Kasper Kirschner, Joachim Knappe, Daniel Lane, Eckehard Lorch, Michio Matsuhashi, Shosaku Numa, Dieter Oesterhelt, Franco Piccinini, Erika Ringelmann and Eckhart Schweizer.

I have attempted to demonstrate how the intense investigation of the biotin-dependent carboxylation reactions led us to the discovery of the multienzyme complex of fatty acid synthesis. We have always considered that our investigations into the detailed biochemical reactions of this enzyme system were important, not only to our understanding of the mechanism of fatty acid synthesis, but of other morphologically defined multienzyme processes as well. To this goal I feel that our endeavours are justifiable.

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