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## The pathway from "activated acetic acid" to the terpenes and fatty acids

*Nobel Lecture, December 11, 1964*

The extreme distinction conferred by the Royal Caroline Institute on the work of Konrad Bloch and myself "On the Mechanism and Regulation of the Cholesterol and Fatty Acid Metabolism" places on me the honoured duty of presenting a report on my investigations to this assembly.

My first contact with dynamic biochemistry in 1937 occurred at an exceedingly propitious time. The remarkable investigations on the enzyme chain of respiration, on the oxygen-transferring haemin enzyme of respiration, the cytochromes, the yellow enzymes, and the pyridine proteins had thrown the first rays of light on the chemical processes underlying the mystery of biological catalysis, which had been recognised by your famous countryman Jöns Jakob Berzelius. Vitamin B<sub>12</sub>, which is essential to the nourishment of man and of animals, had been recognised by Hugo Theorell in the form of the phosphate ester as the active group of an important class of enzymes, and the fermentation processes that are necessary for Pasteur's "life without oxygen" had been elucidated as the result of a sequence of reactions centered around "hydrogen shift" and "phosphate shift" with adenosine triphosphate as the phosphate-transferring coenzyme. However, 1,3-diphosphoglyceric acid, the key substance to an understanding of the chemical relation between oxidation and phosphorylation, still lay in the depths of the unknown. Nevertheless, Otto Warburg was on its trail in the course of his investigations on the fermentation enzymes, and he was able to present it to the world in 1939. It was an extremely exciting year for a young biochemist.

This was the period in which I carried out my first independent investigation<sup>1</sup>, which was concerned with the metabolism of yeast cells after freezing in liquid air, and which brought me directly into contact with the mechanism of alcoholic fermentation. This work taught me a great deal, and yielded two important pieces of information. The first was that in experiments with living cells, special attention must be given to the permeability properties of the cell membranes, and the second was that the adenosine polyphosphate system plays a vital part in the cell, not only in energy transfer, but also in the regula-

tion of the metabolic processes<sup>2</sup>. This investigation aroused by interest in problems of metabolic regulation, which led me to the investigation of the Pasteur effects, and has remained with me to the present day.

«Activated Acetic Acid»

My subsequent concern with the problem of the acetic acid metabolism arose from my stay at Heinrich Wieland's laboratory. Workers here had studied the oxidation of acetic acid by yeast cells, and had found that though most of the acetic acid undergoes complete oxidation, some remains in the form of succinic and citric acids<sup>4</sup>. The explanation of these observations was provided by the Thunberg-Wieland process, according to which two molecules of acetic acid are dehydrogenated to succinic acid, which is converted back into acetic acid via oxaloacetic acid, pyruvic acid, and acetaldehyde, or combines at the oxaloacetic acid stage with a further molecule of acetic acid to form citric acid (Fig. 1). However, an experimental check on this view by a Wieland's student Robert Sonderhoffs brought a surprise. The citric acid formed when trideuteroacetic acid was supplied to yeast cells contained the expected quantity of deuterium, but the succinic acid contained only half of the four deuterium atoms required by Wieland's scheme.

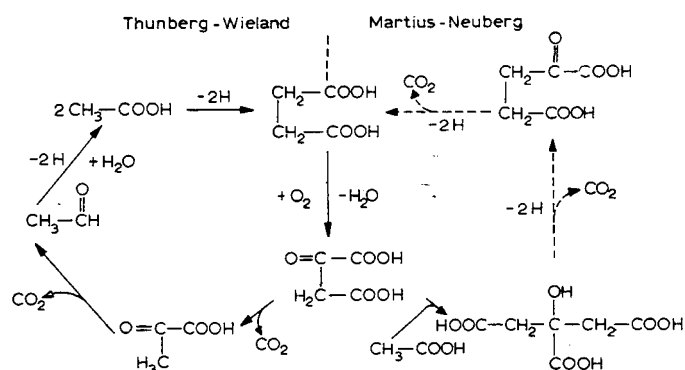


Fig. 1. Views on the degradation of acetic acid in yeast.

The solution to the problems that this raised was provided by Carl Martiu. He realised that citric acid in the animal metabolism enters into equilibrium with isocitric acid and is oxidised to  $\alpha$ -ketoglutaric acid, the conversion of which into succinic acid had already been discovered by Carl Neuberg (Fig. 1).

It was possible to assume with fair certainty from these results that the succinic acid produced by yeast from acetate is formed *via* citric acid<sup>7</sup>. Sonderhoff's experiments with deuterated acetic acid led to another important discovery. In the analysis of the yeast cells themselves, it was found that while the carbohydrate fraction contained only insignificant quantities of deuterium, large quantities of heavy hydrogen were present in the fatty acids formed and in the sterol fraction. This showed that fatty acids and sterols were formed directly from acetic acid, and not indirectly via the carbohydrates. As a result of Sonderhoff's early death, these important findings were not pursued further in the Munich laboratory. This situation was elucidated only by Konrad Bloch's isotope experiments, on which he himself reports.

My interest first turned entirely to the conversion of acetic acid into citric acid, which had been made the focus of the aerobic degradation of carbohydrates by the formulation of the citric acid cycle by Hans Adolf Krebs<sup>8</sup>. Unlike Krebs, who regarded pyruvic acid as the condensation partner of acetic acid, we were firmly convinced, on the basis of the experiments on yeast, that pyruvic acid is first oxidised to acetic acid, and only then does the condensation take place. However, our attempts to achieve the synthesis of citric acid from oxaloacetic acid and acetic acid with the aid of yeast cells whose membranes had been made permeable to polybasic acids by freezing in liquid air were unsuccessful<sup>7</sup>.

Further progress resulted from Wieland's observation<sup>9</sup> that yeast cells that had been "impoverished" in endogenous fuels by shaking under oxygen were able to oxidise added acetic acid only after a certain "induction period" (Fig. 2). This "induction period" could be shortened by addition of small quantities of a readily oxidisable substrate such as ethyl alcohol, though propyl and butyl alcohol were also effective. I explained this by assuming that acetic acid is converted, at the expense of the oxidation of the alcohol, into an "activated acetic acid", and can only then condense with oxalacetic acid<sup>10</sup>.

In retrospect, we find that I had come independently on the same group of problems as Fritz Lipmann<sup>11</sup>, who had discovered that inorganic phosphate is indispensable to the oxidation of pyruvic acid by lactobacilli, and had detected acetylphosphate as an oxidation product. Since this anhydride of acetic acid and phosphoric acid could be assumed to be the "activated acetic acid", I carried out a chemical synthesis of the crystalline silver salt of acetylphosphoric acid<sup>12</sup>. However, the experiment was again unsuccessful; no citric acid was obtained on incubation of acetylphosphate with oxaloacetic acid and yeast preparations. My work on this problem stopped at this stage for a number of

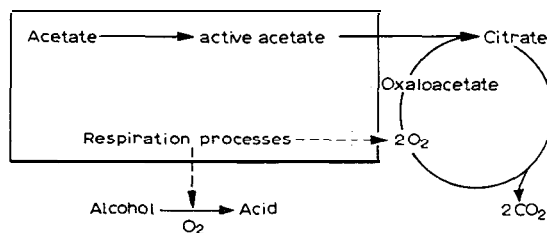
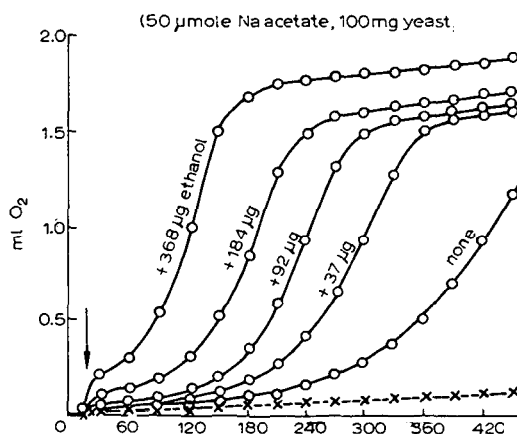


Fig. 2. Effect of alcohol on the «induction time» for the degradation of acetic acid.

years, largely because of the difficulties that surrounded free research during this period.

When scientific contact with the outside world was restored after a break of several years, I learned of the advances that had been made in the meantime in the investigation of the problem of "activated acetic acid". Fritz Lipmann has described the development at length in his Nobel Lecture's, and I need not repeat it. The main advance was the recognition that the formation of "activated acetic acid" from acetate involved not only ATP as an energy source, but also the newly discovered coenzyme A, which contains the vitamin pantothenic acid, and that "activated acetic acid" was probably an acetylated coenzyme A.

Experiments that I carried out with Reichert and Rueff<sup>14,15</sup> on yeast cells agreed well with such a view. We found that during the "induction time" that introduces the oxidation of acetic acid by "impoverished" yeast (Fig. 2), the respiration intensity and the coenzyme A content are proportional to each other. When the oxidation of acetic acid was in full progress, the coenzyme A

content of the yeast had reached a high plateau. We therefore assumed that yeast cells in this phase must also be rich in acetylated coenzyme A, and would be a suitable starting material for its isolation. We might not have attempted to isolate acetyl-CoA but for the fact that I was already possessed by the idea that it was a thio ester. I can still remember exactly how this notion came to me. It was one evening after a conversation with my friend Theodor Wieland, in which we had spent some hours discussing the group of the pantothenic acid component in coenzyme A to which the acetic acid residue might be bound, but without reaching any conclusion. On the way home, it suddenly occurred to me that Lipmann<sup>16</sup> had mentioned the content of disulfides in his publication on coenzyme A, but had not given it any further attention. This was not surprising, since the coenzyme A preparation investigated was by no means pure. However, this remark interested me, since I had been struck by the fact that every single coenzyme A-dependent enzyme reaction known at that time required the addition of cysteine or glutathione. I combined the two observations in the assumption that sulphur was an essential constituent of coenzyme A and could affect the functions of the coenzyme only as a thiol group.

We were therefore not particularly surprised when "activated acetic acid" was found, in the course of purification from yeast extract, to be a thio ester *i. e.* an acylmercaptan of acetic acid and coenzyme A<sup>14,15</sup>. It was identified in various ways, as is described in detail elsewhere<sup>15,17</sup>. I shall confine myself here to mentioning a single piece of evidence, since this involved the use of a new calorimetric method. Thio esters undergo a delayed colour reaction with sodium nitroprusside and alkali, which can be quantitatively evaluated<sup>18</sup>. When we compared the content of "activated acetic acid" as determined by the enzymatic acetylation of sulphanilamide, with the thio ester content in various preparations in the course of purification, we found perfect agreement between the two values (Fig. 3).

The sulphur content of coenzyme A had been verified in the meantime by the work of Snell *et al.*<sup>19</sup>, and it had been found that the thiol group in coenzyme A is present as pantetheine, the peptide of pantothenic acid and cysteamine. Fig. 4 shows the formula of "activated acetic acid" as found in the elucidation of the constitution of coenzyme A, which was described by Lipmann's in his Nobel Lecture.

Following the isolation of "activated acetic acid" and its identification with acetyl-CoA, we next showed that this was the long-sought acetyl donor, which provides the acetic acid residue not only for the acetylation of sulpha-

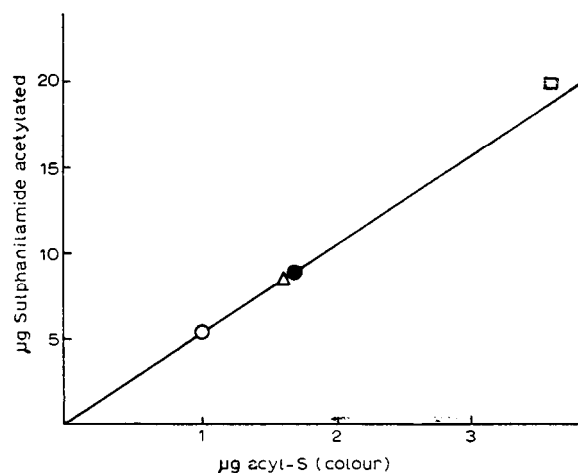


Fig. 3. Identity of acetyl donor and thio ester.  $\circ$ , decomposed barium salt;  $\bullet$ , elution of active charcoal;  $\Delta$ ,  $\square$ , various dry preparations (after precipitation with acetone).

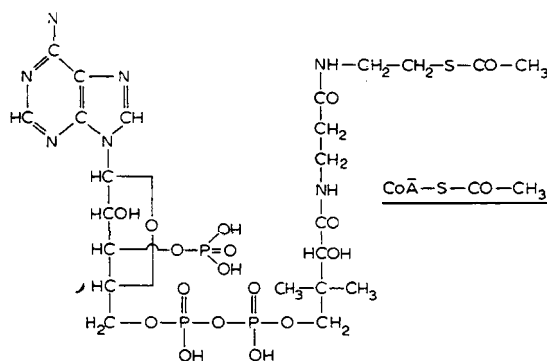


Fig. 4. Formula of «activated acetic acid» = acetyl-CoA.

nil-amide but also for the formation of acetylcholine, citric acid, and acetoacetic acid (*cf.* ref. 17). These experiments chemically elucidated the function of coenzyme A as an acetyl-transferring agent (Fig. 5). It is based on the reversible bonding of the acetyl residue to the thiol group of the coenzyme. This bonding provides the acetyl group with the dual chemical reactivity required for its diverse metabolic reactions, *i. e.* reactivity in the carbonyl group with nucleophilic substituents and reactivity in the neighbouring methyl group with electrophilic substituents<sup>21-23</sup>.

The transfer of the acetyl group from the donor to the acceptor *via* specific enzymes is subject to the same rules as the transport of hydrogen and of phosphate, which was already well known at the time of these investigations<sup>24</sup>. It

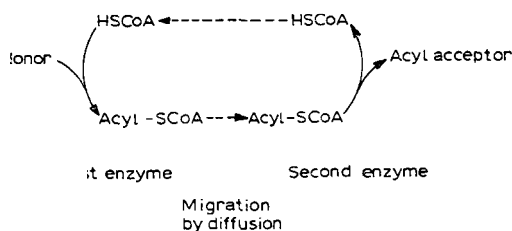


Fig. 5. Scheme of the enzymatic acyl transfer.

is also important in the acetyl transfer that the bonding energy in Lipmann's sense<sup>20</sup> should be retained on transition from the donor to the coenzyme A. In other words, the "bonding energy" of the thio ester acetyl-CoA must be of the same order of magnitude as that of acetylphosphate or of ATP's, as was later fully confirmed by accurate measurements (*cf.* ref. 23). This discovery was of great importance since it meant that the so-called "energy-rich phosphate bond" lost its special place in the energy balance of the cell, and it led to the expectation that other "energy-rich" compounds as well as the "energy-rich" phosphates and thio esters occur in the intermediate metabolism. This assumption has now been confirmed many times over.

### *Fatty Acid Cycle*

The recognition that acetyl-CoA is a thio ester was of the utmost importance in another respect. It was already known from investigations on the degradation of fatty acids by mitochondria from liver or bacterial extracts that the acids must be "activated" before the  $\beta$ -oxidation can begin (*cf.* ref. 25). Moreover, the study of the breakdown of acetoacetic acid and higher  $\beta$ -keto acids had shown that citric acid is formed, and that the path thus leads *via* "activated acetic acid" (*cf.* ref. 26). The discovery of the thio ester linkage in acetyl-CoA enabled me to formulate a chemical reaction scheme for the oxidation of fatty acids that was compatible with all the previous findings<sup>14,15,27</sup>. It was found that the Knoop-Dakin formulation of the  $\beta$ -oxidation<sup>28,29</sup> could be retained in principle if the free acids were replaced by their coenzyme A derivatives (Fig.6). The key reaction of the entire degradation sequence, which I named the "fatty acid cycle", and which is preceded by dehydrogenations and a hydration, consists in the thiolytic cleavage of the  $\beta$ -keto acid by coenzyme A. This yields acetyl-CoA and the coenzyme A derivative of a fatty acid that

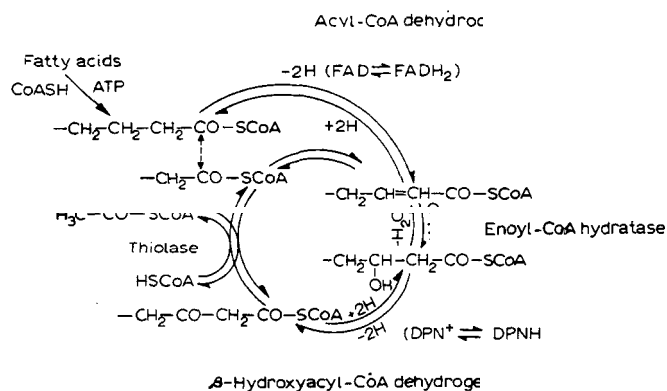
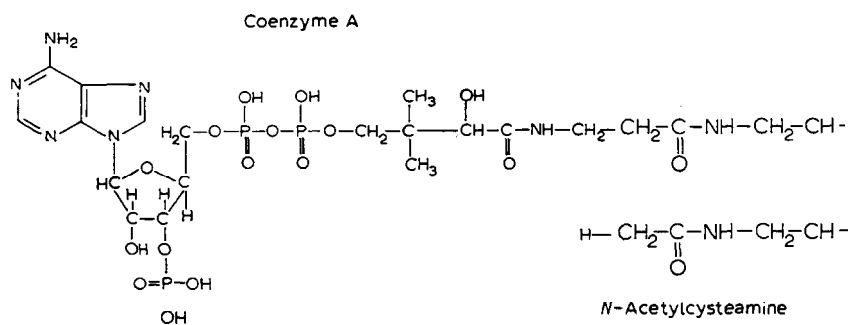


Fig. 6. The «fatty acid cycle»:

contains two carbon atoms less than the original acid, and which is immediately oxidised in the same manner in the next cycle. This reaction sequence can be repeated until the fatty acid has been entirely converted into acetyl-CoA.

The substrate is regenerated in this repeated reaction sequence, which thus resembles the citric acid cycle or the cyclic process in the synthesis of urea. In these other cyclic processes, however, identical substrates are formed after each cycle, whereas a shorter homologous chain is formed in each repetition of the fatty acid cycle. It would therefore be more appropriate to describe the oxidation of fatty acids by a spiral process rather than by a cycle.

When I embarked on the study of the four reaction steps of the fatty acid cycle together with Wessely, Wieland, Seubert, and Reuff, we first synthesised model compounds in which the complicated coenzyme A was replaced by the simple component *N*-acetylcysteamine (Fig. 7) in order to acquaint ourselves with the properties of the assumed intermediate products. We dis-

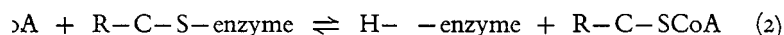
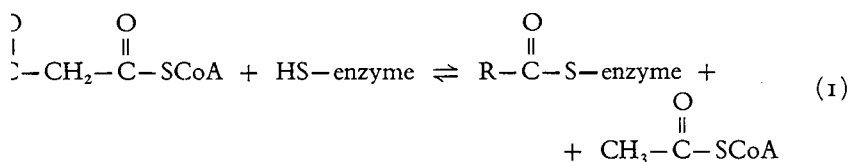
Fig. 7. *N*-Acetylcysteamine, a component of coenzyme A,



covered the specific UV absorption bands of the thio esters of  $\alpha,\beta$ -unsaturated acids and of  $\beta$ -keto acids, which became an indispensable analytical tool in enzyme experiments<sup>21,30</sup>. However, the model substances were also extremely valuable in another respect, since we found that most enzymes of the fatty acid cycle could act on the simple models instead of the natural substrates containing CoA<sup>21,31</sup>. We devised optical enzyme tests on this basis, and this led again to the confirmation of the prognosis of Otto Warburg<sup>24</sup>, the discoverer of this method, that "when followed by an optical test, no enzyme can escape isolation".

At the start of our investigations on the enzymes of the fatty acid cycle, no coenzyme A derivative was known except acetyl-CoA, which we isolated from yeast extract, but which could also be prepared enzymatically from acetylphosphate according to Stadtman<sup>32</sup>. We still lacked the generally applicable chemical preparation methods that were later furnished by Simon and Shermin<sup>33</sup>, by Wieland and Rueff<sup>34</sup>, and by Decker and myself<sup>35,36</sup>. Another method for the preparation of CoA derivatives makes use of specific enzymes, the enzymes of the "fatty acid activation" having proved to be particularly suitable (*cf.* ref. 3 6).

While we were working on the fatty acid cycle, parallel investigations were being carried out by Ochoa and by Green (*cf.* ref. 37). These studies showed that the hydrogen is taken over in the two oxidation steps by flavine adenine dinucleotide and by diphosphopyridine nucleotide, and that L-(+)-hydroxybutyryl-CoA and its homologues are formed in the hydration step (Fig. 6). In the study of thiolase, which is the key enzyme of the "fatty acid cycle" and is responsible for the removal of acetyl-CoA, evidence had been found that a thiol group is present in the active center of the enzyme. My assumption that this takes part in the catalytic process in the sense of Eqns. (1) and (2)<sup>21</sup> has recently been proved by Gehring and myself<sup>38,39</sup>.



It was also shown that the active thiol group belongs to a cysteine residue in the polypeptide chain of the enzyme. This thiolase was finally crystallised in

the pure state from pig's heart (Fig. 8). It has not yet been conclusively established whether the substrates with various chain lengths are acted upon by different enzymes in the thiolase reaction and in the dehydrogenation of the saturated fatty acid-CoA compounds, or whether a single enzyme is sufficient as in the case of  $\beta$  - hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase (*cf.* ref. 36).



Fig. 8. Crystalline thiolase from pig's heart.

In connection with our studies on the degradation of fatty acids, we have also investigated the chemical mechanism of the formation of acetoacetic acid in the liver, which is responsible for the development of ketosis in diabetes mellitus and on starvation. In the study of this process, we encountered a new metabolic cycle, which we called the HMG-CoA cycle<sup>40</sup>, and which must always start to function when the citric acid cycle of the liver can no longer cope with the quantities of acetyl-CoA formed by the degradation of fatty acids. The causes of this insufficiency of the citric acid cycle have been successfully studied in particular by Otto Wieland<sup>41</sup>.

The conversion of acetyl-CoA into acetoacetic acid is introduced by the condensation of acetyl-CoA with itself, which leads, after elimination of coenzyme A, to acetoacetyl-CoA, and is the reversal of the thiolytic cleavage of  $\beta$ -keto acids discussed earlier (Fig. 9). The shortest route from acetoacetyl-CoA to free acetoacetic acid would be hydrolysis by water, which was initially discussed<sup>42,43</sup>. Together with Henning, Bublitz and Sörbo, however, I then found that the process is more complicated. Acetoacetyl-CoA con-

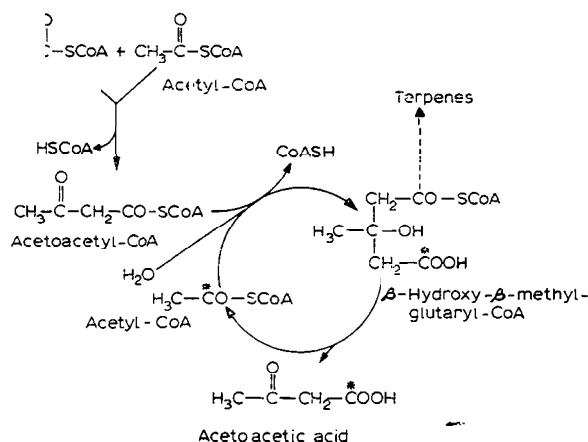


Fig. 9. Formation of acetoacetic acid in the "HMG-CoA cycle".

denses first with acetyl- CoA to form  $\beta$  -hydroxy- $\beta$  -methylglutaryl-CoA, which then gives free acetoacetate and acetyl-CoA in the cleavage reaction discovered by Coon<sup>44</sup> (Fig. 9). A very important condition for the operation of this cycle is that the condensation of acetyl-CoA and acetoacetyl-CoA is practically irreversible, as was shown in experiments by Rudney<sup>45</sup> and by ourselves<sup>40</sup>. This enables the extremely small quantities of acetoacetyl-CoA that exist in enzymatic equilibrium with acetyl-CoA to be intercepted by condensation and the entire reaction drawn in the direction of the  $\beta$  -hydroxy- $\beta$  -methylglutaryl-CoA synthesis (Fig. 10).

It is particularly interesting to note that the cell introduces the liberation of acetoacetic acid from acetoacetyl-CoA, which may be regarded as the degradation process, with the synthesis of  $\beta$  -hydroxy- $\beta$  -methylglutaryl- CoA, and so gains an intermediate that serves as a structural material for the large class of natural products, the terpenes. The "HMG-CoA cycle" may be

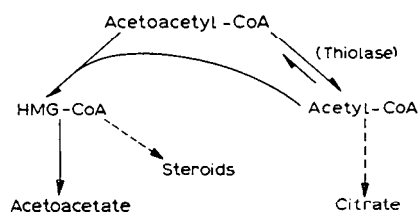
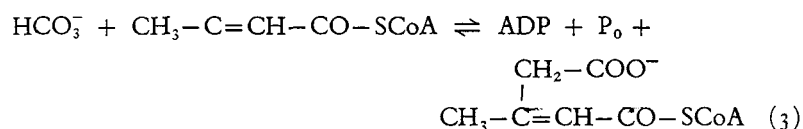


Fig. 10. Importance of the irreversible condensation reaction to the synthesis of carbon chains from acetyl-CoA with thiolase.



was found to be reversible, as was the subsequent decarboxylation. The groundwork for the study of this reaction and of the reverse reaction, *i.e.* the carboxylation of  $\beta$ -methylcrotonyl-CoA had been laid by the investigations of Coons'. These had shown the participation of ATP, but had led to incorrect results regarding the CO<sub>2</sub> acceptor and the cleavage products of ATP.

To set matters right we found that the carboxylation can be described by Eqn.(3).



In order to explain the chemical reactions that connect the cleavage of ATP and the carboxylation, we started with the obvious assumption of an "activated carbon dioxide", a concept that goes back to the fundamental discoveries of Harland Wood<sup>52</sup>. The existence of the compounds carboxyl adenylate<sup>51</sup> or carboxyl phosphate<sup>53</sup> that had been proposed by other authors, on the other hand, seemed very doubtful to us on chemical grounds. An important clue to the direction in which to seek for the solution of the problem of "activated carbon dioxide" was provided by earlier investigations by Lardy<sup>54</sup>, in which a series of CO<sub>2</sub> fixation reactions had been found to be impaired by a deficiency of biotin. However, all attempts to detect biotin as a component of carboxylating enzymes had previously been unsuccessful. Tietz and Ochoa<sup>55</sup> had even looked for biotin in a highly purified preparation of propionyl-CoA carboxylase, the action of which was similar to that of our carboxylase, but had found nothing.

Nevertheless, I could not rid myself of the beliefs that biotin was the active group of our  $\beta$ -methylcrotonyl-CoA carboxylase and that the "activated carbon dioxide" was a carboxylated biotin. In collaboration with Knappe, Lorch and Ringelmann<sup>50, 56</sup>, I first succeeded in showing by quantitative biotin determinations that all the active preparations of our carboxylase contained covalently bound biotin. Moreover, the enzyme activity and the biotin content increased in the same ratio during the purification of the enzyme (Fig. 12). The identity of biotin with the active group of the carboxylase was shown by the specific inhibition of the enzyme by avidin from raw egg-white. Avidin combines with biotin to form an extremely stable complex, and so blocks the catalysis. This simple method of characterising enzymes that con-

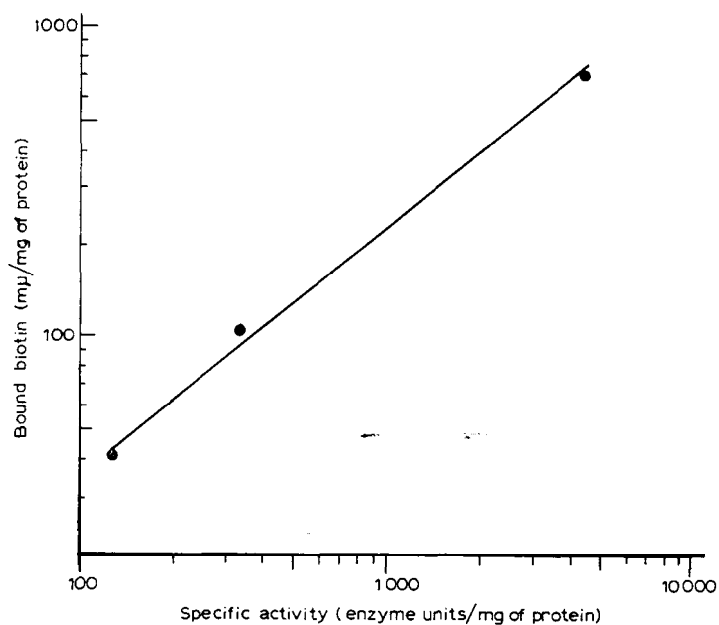
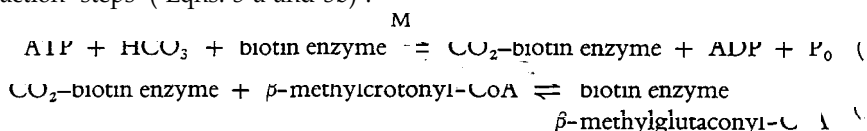


Fig. 12. Proportionality between enzyme activity and biotin content.

tain biotin by inhibition with avidin is based on investigations by Wessman and Werkman<sup>57</sup>. It was recently used again for the first time by Wakil in the investigation of the synthesis of fatty acids<sup>58</sup>.

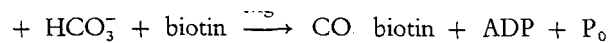
By exchange experiments with labelled substrates, we were then able to subdivide the carboxylation of  $\beta$ -methylcrotonyl-CoA into the following reaction steps<sup>59</sup> (Eqns. 3 a and 3b):



In these investigations, a direct indication of the  $\text{CO}_2$ -biotin enzyme was provided in particular by the avidin-inhibited isotope exchange between  $\beta$ -methylcrotonyl-CoA and radioactive  $[1,3,5\text{-}^{14}\text{C}]$ -methylglutaconyl-CoA. The general validity of this reaction course was subsequently demonstrated on carboxylases of various specificities, initially by exchange experiments similar to those developed by us in the study of  $\beta$ -methylcrotonyl-CoA carboxylase, but later also by direct isolation of the  $\text{CO}_2$ -biotin enzymes<sup>59,60</sup>. The first report on this was by Kaziro and Ochoa<sup>61</sup>, who repeated the biotin determination, after the publication of our results, on propionyl-CoA car-

boxylase, which had been crystallised in the meantime, and who now found that biotin was also an essential component of the enzyme.

After the completion of the exchange experiments on methylcrotonyl-CoA carboxylase, which confirmed our concept of the existence of "activated carbon dioxide" in the form of the CO<sub>2</sub>-biotin enzyme, we had turned immediately to the investigation of the nature of the bond between carbon dioxide and biotin. The carboxylase proved a great success in these investigations. In view of the good results that I had obtained with model substrates in the study of the enzymes of the fatty acid cycle, we used free biotin in the carboxylase reaction. It was found that the enzyme then used free biotin instead of the specific substrate β - methylcrotonyl- CoA in the carboxylation process, and gave a carboxylated biotin<sup>50,56</sup> in accordance with Eqn. (4).



This reaction is unusual in that it had not been observed with any other enzyme. On the other hand, its specificity (only natural (+) - biotin reacts) made it reasonable to assume that it was a model for the reaction step shown in Eqn. (3a), with free biotin in place of the enzyme-bound biotin.

The explanation in our view is that the biotin active group of the enzyme is bonded to the protein not only *via* the carboxyl group but also *via* a second centre, possibly the ring system (Fig. 13). Free biotin also has an affinity for this second bonding site, so that when introduced in higher concentrations, it displaces the active group of the enzyme and becomes charged with carbon dioxide in its place. In this way, we obtained the radioactive <sup>14</sup>CO<sub>2</sub>-biotin, which proved to be extremely unstable to acids, in experiments with radioactive [<sup>14</sup>C] bicarbonate. This was not surprising, since I had assumed from chemical considerations that the active centre was the imidazolidone ring of biotin. To remove excess [<sup>14</sup>C] bicarbonate that was already present in the reaction mixture in these experiments without destroying the unstable <sup>14</sup>CO<sub>2</sub>-biotin, we used a simple experimental trick. We passed normal <sup>12</sup>CO<sub>2</sub> through the reaction mixture and so completely eliminated the radioactive <sup>14</sup>CO<sub>2</sub>, whereas the radioactive <sup>14</sup>CO<sub>2</sub>-biotin remained behind. As can be seen from Table 1, the controls without ATP or without biotin no longer exhibited any activity under the same conditions.

It was then a purely chemical problem to stabilise the labile radioactive carboxybiotin by conversion into the methyl ester with diazomethane and to identify the stable product as 1'-N-carbomethoxybiotin methyl ester by comparison with synthetic compounds<sup>62</sup> and by an X-ray analysis carried out

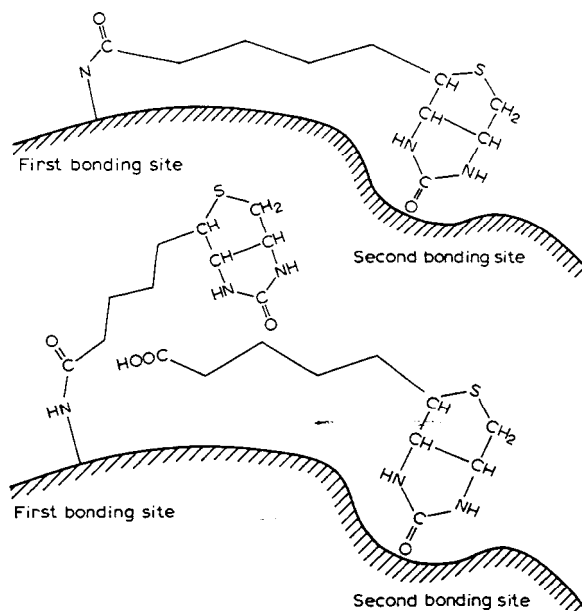


Fig. 13. Hypothetical mechanism of the bonding of free biotin to the carboxylase.

by Steinrauf in Urbana<sup>63</sup>. My colleague Knappe showed a great deal of personal initiative in the solution of this problem. We were thus able to assign the partial formula shown in Fig. 14 to the "activated carbon dioxide". Some uncertainty that still remained after these experiments regarding the constitution of the  $\text{CO}_2$ -biotin enzymes has now been cleared up by the elucidation of the  $\text{CO}_2$  binding sites in four different carboxylated biotin enzymes themselves.

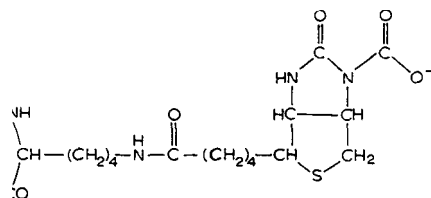
Table 1  
Fixation of  $^{14}\text{CO}_2$  by (+)-biotin

The complete reaction mixture consisted of 20  $\mu\text{moles}$  of Tris buffer, pH 8.2,  $\mu\text{moles}$  of  $\text{MgSO}_4$ , 20  $\mu\text{moles}$  of (+)-biotin, 0.5  $\mu\text{moles}$  of ATP, 3.3  $\mu\text{moles}$  of  $\text{KH}^{14}\text{CO}_3$  (10<sup>5</sup> counts/min), and 50  $\mu\text{g}$  of carboxylase (Mycobact. spec. act. 400) in a total volume of 0.5 ml. The mixtures were incubated for 60 min at 37°, then cooled to 0°, and a rapid current of  $\text{CO}_2$  was passed through for 20 min. Aliquot parts of the solution were evaporated to dryness and counted for the radioactivity measurement.

	Complete	Without ATP	Without biotin
Counts/min	130 000	0	0
$\mu\text{moles}$ of $^{14}\text{CO}_2$ fixed <sup>a</sup>	0.43	0	0

<sup>a</sup> Calculated from the radioactivity fixed.





14. Structure of «activated carbon dioxide».

Knappe<sup>62a</sup>, who had moved in the meantime to the University of Heidelberg, where he devised the methods, as well as Lane<sup>64</sup>, Wood<sup>65</sup>, and Numa<sup>66</sup> in my own laboratory had taken part in these investigations. The bonding between biotin and the protein always takes place *via* the  $\epsilon$ -amino group of peptide-bound lysine residues, as can be seen from Fig. 14. This bonding site, which had been known for a long time from biocytin, was first detected in propionyl-CoA carboxylase by Kosow and Lane<sup>67</sup>. The biotin enzymes that have been discovered so far are listed in Table 2

Though the primary structural problem of "activated carbon dioxide" has now been solved, questions still remain concerning its bonding and reactivi-

Table 2  
of biotin enzymes known at present and their substrates

Biotin enzyme:	Substrate:
Acetyl-CoA carboxylase	$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{SCoA}$
Propionyl-CoA carboxylase	$\text{CH}_3-\text{CH}_2-\overset{\text{O}}{\parallel}{\text{C}}-\text{SCoA}$
$\beta$ -Methylcrotonyl-CoA carboxylase	$\text{CH}_3-\overset{\text{CH}_3}{\underset{ }{\text{C}}}=\text{CH}-\overset{\text{O}}{\parallel}{\text{C}}-\text{SCoA}$
Geranyl-CoA carboxylase	$\text{R}-\overset{\text{CH}_3}{\underset{ }{\text{C}}}=\text{CH}-\overset{\text{O}}{\parallel}{\text{C}}-\text{SCoA}^*$
Pyruvate carboxylase	$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{COOH}$
Methylmalonyl-CoA-pyruvate transcarboxylase	$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{COOH}$ or $\text{CH}_3-\text{CH}_2-\overset{\text{O}}{\parallel}{\text{C}}-\text{SCoA}$

$\text{CH}_3$

\*  $\text{R} = \text{CH}_3-\text{C}=\text{CH}-\text{CH}_2-\text{CH}_2$

ty<sup>59,68</sup>. Unfortunately, I cannot discuss these problems further here. I must content myself with saying that the activation of carbon dioxide in the CO<sub>2</sub>-biotin enzymes is due to the withdrawal of electrons by the ureido system, which is responsible for the electrophilic character of the carbonyl C atom and hence for the transacylation properties. The acceptors of the "activated carbon dioxide" are methyl or methylene groups that tend to form carbanions under the influence of neighbouring carbonyl groups (*cf.* Table 2). According to experiments by Rétey, the steric configuration is retained on carboxylation of propionyl-CoA. This means that the entering carboxyl group takes up the position abandoned by the leaving proton. Moreover, it is very probable that the transcarboxylation follows a quasi-six-membered ring mechanism, as John R. Johnson pointed out to me following a lecture at Cornell University - (Fig. 15).

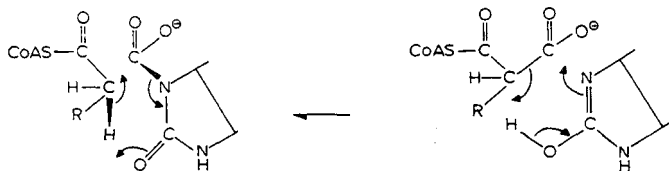


Fig. 15. Mechanism of the CO<sub>2</sub> transfer.

The reactivity of the biotin-bound carbon dioxide is also expressed in the energy relations. The free energy of the decomposition of CO<sub>2</sub>-biotin enzyme was found by Wood in my laboratory to be  $F^{0'} = -4.74$  (kcal/mole) at pH 7.0. The CO<sub>2</sub>-biotin enzymes thus fall among the "energy-rich" compounds of the metabolism, though at the lower end of this range of compounds. The CO<sub>2</sub>biotin enzymes are even more unstable than free CO<sub>2</sub>-biotin, and this shows that the protein part of the biotin enzymes also contributes considerably to the reactivity of "activated carbon dioxide"<sup>68</sup>. The nature of this contribution is not yet known; its determination will require an insight into the arrangement of the amino acids in the "active centre" of the biotin enzymes.

### *The Biosynthesis of Terpenes*

As I shall show later, the careful study of the biotin enzymes has contributed greatly to our understanding of the biological fatty acid synthesis. The di-

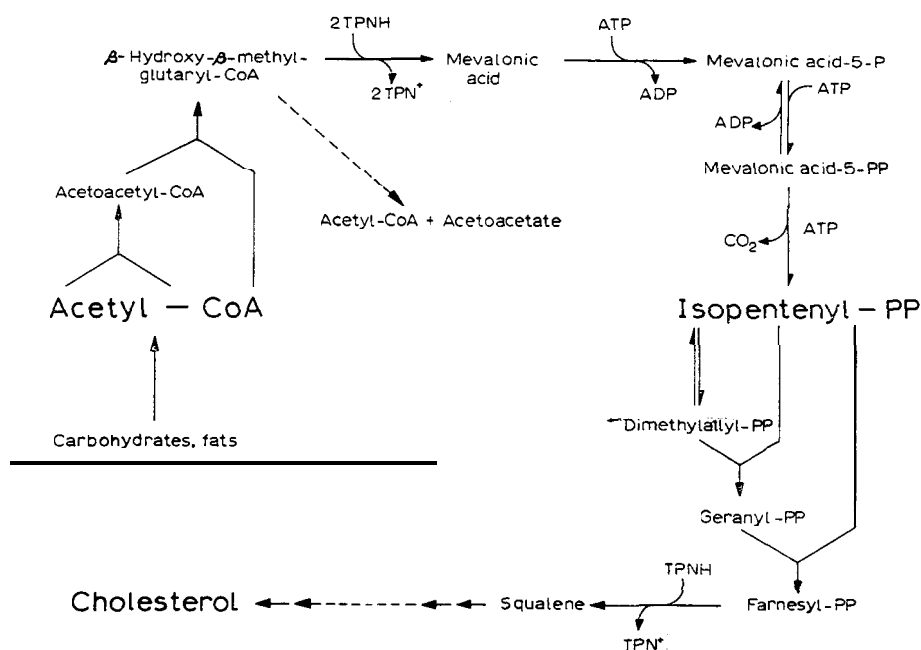


Fig. 16. Pathway of the biosynthesis of terpenes.

rected experiments were of no importance to the elucidation of the conversion of acetyl-CoA into terpenes and into cholesterol, which was the original purpose of our investigations on  $\beta$ -methylcrotonyl-CoA carboxylase. It was in fact found that  $\beta$ -methylcrotonyl-CoA was not the sought structural component of the terpenes and of cholesterol. The necessary breakthrough was provided by the discovery of mevalonic acid by the research team working with Karl Folkers in the laboratories of Merck; Sharp and Dohme<sup>69</sup>. This discovery led to investigations in which my research team competed with that of Konrad Bloch, and which culminated in the simultaneous identification of "activated isoprene" as  $\Delta^1$ -isopentenylpyrophosphate<sup>70,71</sup>. This work has been described in detail by Konrad Bloch in his Nobel Lecture.

There is therefore no need to describe the course of our investigations in detail, and I can therefore confine myself to throwing a few rays of light on the completed picture of the terpene synthesis. Fig. 16 shows the chain of reaction steps leading from acetyl-CoA to squalene, which can be divided into three sections. In the first section, the carbon skeleton of mevalonic acid is synthesised from three molecules of acetyl-CoA. The energy required for this process is provided by three thio-ester linkages, which are broken with

liberation of coenzyme A, and by the oxidation of the two TPNH molecules, which reduce the  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA to mevalonic acid. Mevalonic acid is converted in the second section into isopentenylpyrophosphate. The formation of "activated isoprene" requires three molecules of ATP and is additionally promoted by the removal of a molecule of  $\text{CO}_2$ . No additional energy source is required for the synthesis of the long carbon chains in the final phase, since sufficient energy is available through the removal of the inorganic pyrophosphate from the allyl compounds. The inorganic pyrophosphate formed probably suffers immediate hydrolysis by pyrophosphatase in the cell medium, and this contributes to the irreversibility of the entire process.

In collaboration with Eggerer, Henning, Kessel, Knappe and Agranoff, I have developed methods for the quantitative determination of each of the enzymes involved and for following their purification. We have thus laid a foundation that may also be of value to the physician. The enzyme for the reduction of  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA demands special attention in this connection. This process is remarkable in that the reduction of the thio ester to the alcohol is practically irreversible and is effected in a single act, so that the aldehyde intermediate does not occur in the free form<sup>72,73</sup>. Since the presence of a thiol group can be shown in the active centre of the enzyme involved, we have assumed that the aldehyde intermediate remains bound to the SH group of the enzyme in the form of the hemimercaptal. However, this assumption has not yet been confirmed experimentally.

The work on the reduced enzyme is of the utmost importance in particular with regard to the physiological regulation of the terpene and cholesterol syntheses. The enzyme is found in the microsome fraction of liver homogenates, and functions in the animal body as the "pacemaker enzyme" for the cholesterol synthesis. Nancy Bucher<sup>74,75</sup> was not only the first to postulate this important point, but also provided experimental support for her view by direct enzyme determinations during her short stay in my laboratory. Her findings were then confirmed by other workers<sup>76,77</sup>.

The regulation of the cholesterol formation at this step of the synthesis chain is very rational, since once the mevalonic acid has been formed in the higher organism, it can only be disposed of in the sterol synthesis, owing to the irreversibility of the reductase reaction; the precursor  $\beta$ -hydroxy- $\beta$ -methylglutaryl-CoA, on the other hand, is reincorporated into the acetyl-CoA pool by cleavage into acetyl-CoA and acetoacetate.

The two other enzymes that I would like to mention are those that we

named isopentenylpyrophosphate isomerase and farnesylpyrophosphate synthetase or polymerase<sup>78,79</sup>. The combined action of these enzymes results in the construction of the carbon chain by electrophilic addition to methylene double bonds. The isomerase, which causes the conversion of isopentenylpyrophosphate into dimethylallylpyrophosphate ( *cf.* Fig. 16), is completely inhibited by thiol reagents; we therefore believe that the active group of the enzyme contains a thiol group, and that the isomerisation takes place via the intermediate addition of the SH enzyme to the double bond and its subsequent elimination. The isomerase plays a very important part in the process of the terpene synthesis, since it "triggers", via the formation of dimethylallylpyrophosphate, the construction of the carbon chains with the cooperation of the polymerase. Once started, this process which results in each step in the formation of the homologous allylpyrophosphate lengthened by one isoprene unit, can carry on, and is limited only by the specificity of the enzyme involved in the polymerisation. The enzyme of yeast practically stops at the farnesylpyrophosphate stage, though geranylgeranylpyrophosphate<sup>80</sup>, the parent substance of the diterpenes, of phytol, and of the carotenoids, may also be formed in a very slow reaction. The polymerising enzyme in the latex of *Hevea Brasiliensis*, on the other hand, carries the process on to the formation of high molecular weight rubber<sup>81</sup>.

It should also be mentioned that the alkylating ability of the allylpyrophosphates is not confined to the double bond of isopentenylpyrophosphate, but can also be exerted on the double bonds of substituted hydroquinones<sup>79</sup> or of protohaemin. This probably explains the formation of the various vitamins K and ubiquinones as well as of the cytohaemin of Warburg's respiratory en-

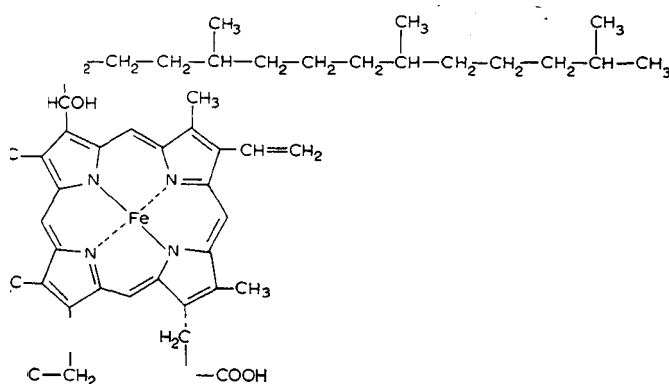


Fig. 17. Chemical structure of cytohaemin.

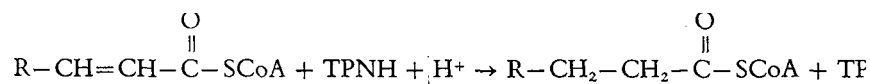
zyme. This iron compound, which is extremely important to aerobic life, and whose discovery was described by Otto Warburg in his Nobel Lecture<sup>82</sup> in 1931, has the structural formula shown in Fig. 17, as I was able to show a year ago together with Grassl and Seyffert<sup>83</sup>.

Our investigations on the biosynthesis of terpenes would not have led to the desired results in such a short time but for the fact that we had available Eggerer's chemical methods<sup>84,85</sup> for the synthesis of isopentenylpyrophosphate and of dimethylallylpyrophosphate and its homologues. Our method for the extraction of allylpyrophosphates from aqueous solutions with colli-dine was also of great value. This method enabled Henning and me to discover the first member of this very acid-sensitive class of compounds of the cell metabolism in farnesylpyrophospha<sup>70,79</sup>.

### *The Multienzyme Complex of the Fatty Acid Synthes*

Encouraged by the successes in the study of the reaction chain of the terpene synthesis, I turned once more to the elucidation of the fatty acid synthesis. I had originally been misled in this field by the assumption, in the formulation of the fatty acid cycle, that the synthesis of the fatty acids was a reversal of the degradation reactions<sup>30</sup>. This belief had been strengthened by the fact that all four reaction steps of the fatty acid cycle are reversible. However, synthetic experiments carried out with this system by Stansley and Beinert<sup>86</sup> in Madison did not yield the expected results.

Following experiments by Langdon<sup>88</sup>, Seubert and I<sup>87</sup> discovered a new enzyme that occurs widely in animal tissues and in yeast, and which transfers hydrogen from reduced triphosphopyridine nucleotide to the CoA derivatives of  $\alpha, \beta$ -unsaturated fatty acids (Eqn. 5); this enzyme is entirely different from the acyl-CoA dehydrogenases that are involved in the fatty acid degradation.



By combination of this new enzyme with purified enzymes of the fatty acid cycle and auxiliary enzymes for the regeneration of reduced diphosphopyridine and triphosphopyridine nucleotides (Fig. 18), it was then possible to incorporate radioactive acetyl-CoA into caproyl-CoA with formation of fatty acids having 8, 10 and more carbon atoms. It is not yet known whether this

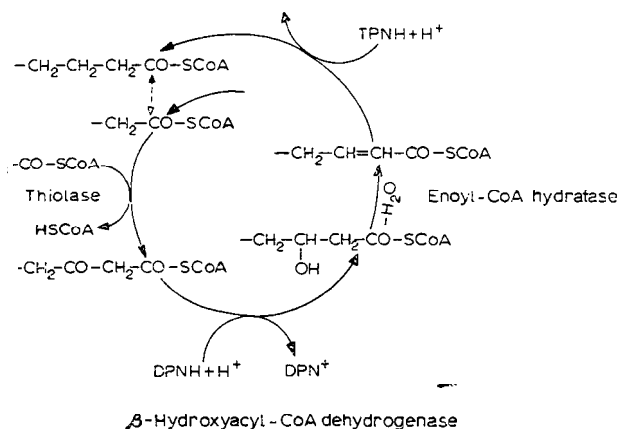
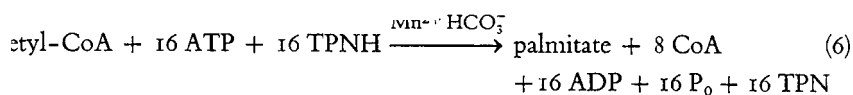


Fig. 18. Synthesis of fatty acids by reversal of the degradation reactions.

system is involved in the synthesis of fatty acids in mitochondria, which contain all the enzymes necessary for a synthesis by this route.

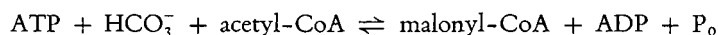
However, this is not the main pathway for the biosynthesis of fatty acids. The foundation for the discovery of this pathway was laid by Wakil's important investigations in Green's laboratory. He and his colleagues isolated two enzyme fractions from pigeons' livers that were capable of synthesising palmitic acid from acetyl-CoA; in addition to TPNH, however, this synthesis required  $ATP^{89,90}$ ,  $Mn^{2+}$  ions, and, most surprising of all, bicarbonate. The effect of the bicarbonate was not concerned with the incorporation of  $CO_2$  into the fatty acid chains. Radioactive carbon from the added  $CO_2$  did not reappear in the palmitic acid isolated. The overall equation for the synthesis was formulated by Wakil's group<sup>91</sup> as follows :



Another peculiarity was the high biotin content of one of the two fractions. This biotin was obviously an essential component, since the fatty acid synthesis was completely inhibited by avidin<sup>88</sup>. However, the function of this first discovered enzyme and the manner in which carbon dioxide is involved in the fatty acid synthesis was at first unknown in Madison.

When Green reported on the state of these investigations at the Gordon Research Conference on Lipid Metabolism on June 12, 1958, I suggested during the discussion of his lecture that the necessity of ATP and carbon dioxide

might be explained by the intermediate formation of malonyl-CoA<sup>22</sup>. Malonyl-CoA could be formed by carboxylation from acetyl-CoA in accordance with Eqn. 7, in analogy with the carboxylations of  $\beta$ -methylcrotonyl-CoA or propionyl-CoA, which had been investigated in detail by that time.

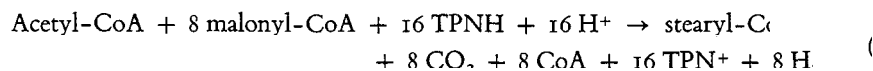


Malonyl-CoA had been shown by Hayaishi<sup>92</sup> to be a very probable intermediate in the bacterial decarboxylation of malonic acid. It seemed very plausible to me on chemical grounds that the same compound might also be an intermediate in the fatty acid synthesis. On the one hand, chemical experience shows that the methylene group of malonyl-CoA should be more nucleophilic and better suited to condensation with thio esters than the methyl group of acetyl-CoA. Moreover, it was possible to predict that CO<sub>2</sub> would be eliminated in the condensation with malonyl-CoA, and this should displace the reaction in favour of the formation of the  $\beta$ -keto acid.

My hypothesis was then confirmed within a short time. Reports were published in quick succession by Brady<sup>93</sup>, who was present at the Gordon Conference, by Wakil<sup>94</sup>, and by my own research group<sup>22</sup> on experiments that conclusively verified the occurrence of malonyl-CoA in the fatty acid synthesis. Wakil<sup>94</sup> showed that the enzyme fraction containing biotin is an acetyl-CoA carboxylase, in excellent agreement with our results for the  $\beta$ -methylcrotonyl-CoA carboxylase<sup>22</sup> as reported earlier.

Wakil's second enzyme fraction is responsible for the conversion of malonyl-CoA into the fatty acids, acetyl-CoA being necessary as the "initiator" of the synthetic reaction in addition to TPNH as the reducing agent<sup>95</sup>. The two C atoms of acetyl-CoA are found at the methyl end of the fatty acids synthesised; the only explanation for this is that the carbon chains are built up by successive addition of C<sub>2</sub> units from malonyl-CoA to acetyl-CoA<sup>96,97</sup>.

We gained an insight into the chemical details of the synthetic process by the study of the pure enzyme from yeast cells. The only fundamental difference between this and the animal enzyme is that a mixture of palmityl-CoA and stearyl-CoA is formed as the product of the synthesis instead of free palmitic acid<sup>96</sup>. The synthesis of the stearyl-CoA can be represented by Eqn. 8.



A puzzling feature in the action of this "fatty acid synthetase" from animal organs or from yeast cells was that the oxidation products known from the



study of the fatty acid degradation, such as the  $\alpha,\beta$ -dehydro-,  $\beta$ -hydroxy-, and  $\beta$ -ketoacyl-CoA compounds, are not intermediates of the synthesis. These products showed no change when added to the purified synthetase in physiological concentrations<sup>95,96</sup>.

The solution to this puzzle was provided by our discovery that thiol groups of the enzyme protein are functionally involved in the process of synthesis, and the intermediates through which the carbon chains are built up are covalently bonded to the enzyme<sup>98,99</sup>. The way for this idea, which came to me after a long period of fruitless experiments, was prepared by our investigations on thiolase or isopentenylpyrophosphate isomerase, which had led us to postulate similar covalent substrate enzyme compounds.

According to our experiments on the highly purified yeast enzyme, the fatty acid synthesis can be described by the scheme in Fig. 19<sup>99-101</sup>. It should be pointed out that at least two types of thiol groups of the enzyme take part in the chemical reactions; to distinguish these groups, we tentatively called them "central" and "peripheral" SH groups. They have different functions, and are distinguished in our reaction scheme by the use of bold and normal type. The synthesis begins with the charging of the enzyme with acetic acid and malonic acid by transfer of the acid residues from acetyl-CoA to the "peripheral" thiol group and from malonyl-CoA to the "central" thiol group. This is followed by the condensation with decarboxylation, which leads to lengthening of the carbon chain, and may therefore be regarded as the heart of the synthesis; this condensation thermodynamically favours the formation of the acetoacetyl-enzyme. The formation of the keto acid is followed by the stepwise conversion into the saturated acid, the saturated butyryl-enzyme being formed *via* reduction by TPNH to the D (-)-B-hydroxybutyryl enzyme, dehydration of the latter to the crotonyl enzyme, and further reduction by TPNH. Theorell's flavine mononucleotide takes part in this second reduction as a hydrogen-transferring coenzyme. All these chemical changes take place on esters that are bonded to the "central" thiol group. At the stage of the butyryl enzyme, the carboxylic acid residue, which has now been lengthened by one C<sub>2</sub>-unit in relation to acetic acid, moves to the "peripheral" SH group, leaving the "central" SH group free to accept a new malonyl residue. The process is repeated until the long-chain fatty acids have been formed. When this stage is reached, the palmityl or stearyl enzyme gives up the saturated fatty acid residue to coenzyme A in the termination reaction, with formation of palmityl-CoA or stearyl-CoA and regeneration of the free enzyme, which can then react once more with acetyl-CoA and malonyl-CoA.

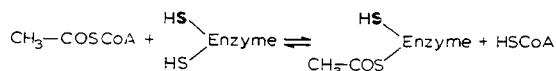
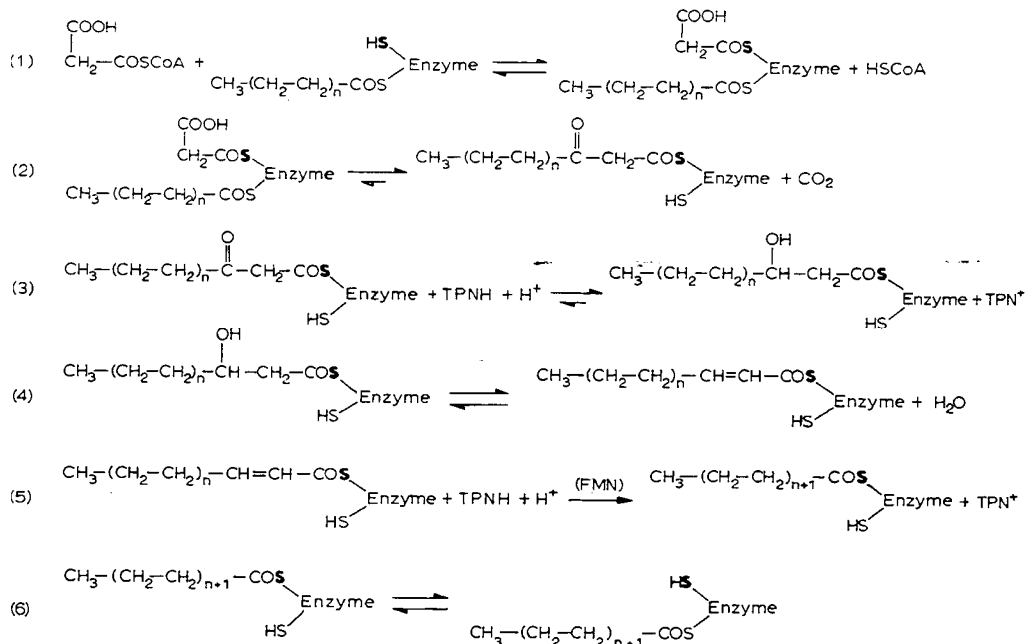
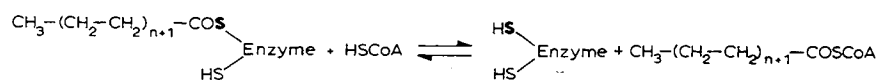
Initiation:Chain-lengthening reactions:Termination:

Fig. 19. Mechanism of the fatty acid synthesis.

This sequence of reaction steps takes place on a multienzyme complex, the structural unit of which we believe to consist of six or seven different enzymes, which are arranged around the "central" SH group (Fig. 20) in such a way that the carboxylic acids bonded to the SH group can come into contact with the various enzymes in succession. Our view is again illustrated in Fig. 21 for the reduction of the  $\beta$ -keto acid to the  $\beta$ -hydroxy acid. The details of the structure of the enzyme complex are largely hypothetical. For example, it is not known whether the central thiol group belongs to a structural element of

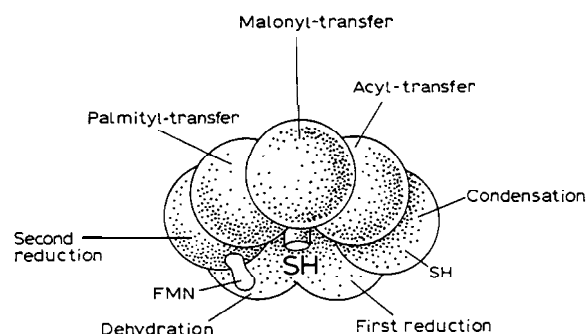


Fig. 20. Hypothetical structure of the multienzyme complex for the fatty acid biosynthesis.

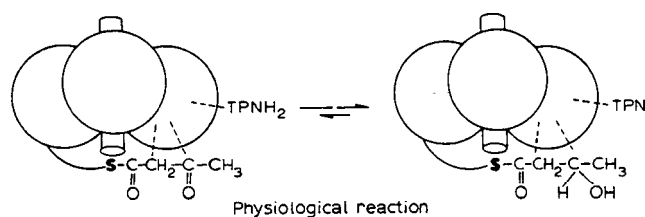


Fig. 21. Model of the reduction of the enzyme-bound  $\beta$ -keto acids by TPNH.

its own, as was assumed in Fig 20, or whether it is covalently bonded to one of the other enzyme components. We prefer the former possibility, since the structure of the fatty acid synthetase from yeast would then correspond to the structure of the analogous enzyme system from *Escherichia coli*, the study of which has recently led Vagelos *et al.*<sup>97</sup> to very good results that confirm our investigations. The multienzyme complex from yeast, unlike the enzyme system of the bacteria is very stable, and the conditions required for cleavage into its components are so severe that the enzymes suffer substantial damage. During the fractionation of the yeast extract by mild methods designed to preserve the activity of the enzymes, the fatty acid synthetase behaves as a single particle, which migrates uniformly in an electric field and in the analytical ultracentrifuge, and has a molecular weight of about 2.3 million. The ordered structure of the multienzyme complex is also visible in the electron microscope (Fig. 22). The photograph, which was supplied by Dr. Hofschneider<sup>102</sup>, shows hollow oval particles surrounded by an equatorial ring. The longer axis of the particles is about 250 Å long, while the shorter axis is about 210 Å long. The electron microscopic structure of the fatty acid synthetase unfortunately cannot be fitted into structural schemes that are known at present.

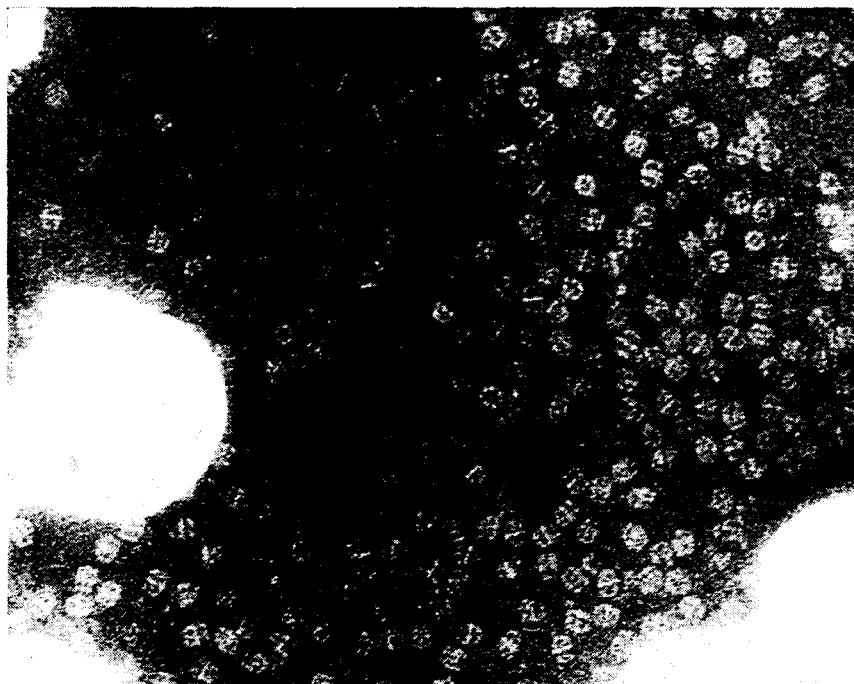


Fig. 22. Electron micrograph of the purified fatty acid synthetase from yeast.

On the basis of our chemical findings, the structure could consist of three rings packed one inside another. This would agree with the assumption that the particles having a molecular weight of 2.3 million consist of three functionally complete sets of enzymes for the fatty acid synthesis; this assumption is also supported by other findings.

I cannot describe here the many experiments that enabled me in collaboration with Duba, Eggerer, Hagen, Kessel, Kirschner, Larch and Schweizer, to clarify the chemical details of the fatty acid synthesis. The model substrates used in the investigation of the enzyme of the fatty acid degradation were again found to be extremely useful. In other experiments, we used the purified fatty acid synthetase as the substrate, and by stoichiometric reaction with acetyl-CoA, malonyl-CoA, and sometimes also TPNH, we were then able to detect the natural intermediates themselves, such as acetyl enzyme, malonyl enzyme, acetoacetyl enzyme, and D (-)-hydroxybutyryl enzyme.

Together with Oesterhelt I have been trying during the past few months to measure the equilibrium constant of the formation of the acetoacetyl enzyme from acetyl-CoA, malonyl-CoA, and enzyme in accordance with Eqn.9,

since the study of this reaction has opened the way to the elucidation of the fatty acid synthesis.

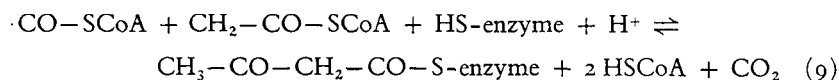
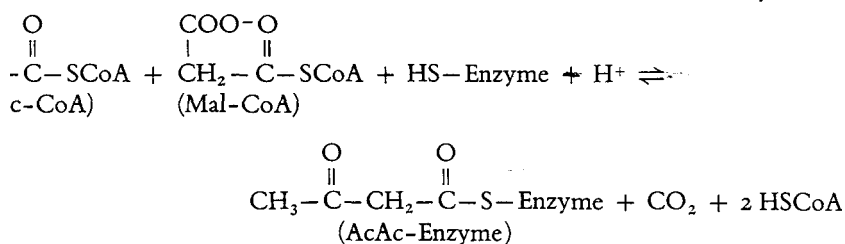


Table 3

mination of the equilibrium constant for the condensation with malonyl-CoA



CoA ( $\mu\text{M}$ )	CO <sub>2</sub> ( $\mu\text{M}$ )	AcAc- Enzyme ( $\mu\text{M}$ )	M ( $\mu\text{M}$ )	oA ( $\mu\text{M}$ )	AcCoA ( $\mu\text{M}$ )	HS-Enzyme ( $\mu\text{M}$ )	H <sup>+</sup> ( $\mu\text{M}$ )	K <sub>eq</sub> × 10 <sup>5</sup>
7.5	339	2.120	59.2	52.7	2.420	0.316	1.06	
7.0	1008	1.810	59.7	53.0	2.725	0.316	2.50	
5.8	1700	1.420	60.0	53.3	3.115	0.316	2.62	
	2380	1.110	60.3	53.7	3.425	0.316	2.62	
	366	1.290	58.7	65.3	0.425	0.316	1.33	
	366	0.880	59.1	65.7	0.840	0.316	1.51	
	366	0.510	59.5	66.1	1.210	0.316	1.75	
	366	0.324	59.7	66.3	1.396	0.316	2.08	
	366	0.222	59.8	66.4	1.498	0.316	2.12	
	366	1.020	59.0	65.6	0.695	0.316	2.14	
	366	1.220	118.8	65.4	0.500	0.316	1.77	

Average: K<sub>eq</sub> = 1.95 · 10<sup>5</sup>

Results of our measurements are presented in Table 3.

Comparison of the value for the equilibrium constant of the condensation with malonyl-CoA :

$$\frac{[\text{Acetoacetyl-enzyme}] [\text{CoA}]^2 [\text{CO}_2]}{[\text{Acetyl-CoA}] [\text{malonyl-CoA}] [\text{enzyme}] [\text{H}^+]} = 1.95 \cdot 10^5 \quad (0^\circ)$$

with the value for the equilibrium constant of the condensation with acetyl-CoA, which also gives a thio ester of acetoacetic acid:

$$K_{\text{eq}} = \frac{[\text{Acetoacetyl-CoA}][\text{CoA}]}{[\text{Acetyl-CoA}]^2} = 1.6 \cdot 10^{-5} \quad (25^\circ, \text{pH})$$

shows the great advantage that results from the use of malonyl-CoA for the construction of the carbon chains. Living nature makes use of this advantage both in the synthesis of fatty acids and in the biosynthesis of many other natural products<sup>101</sup> that come under Birch's polyacetate rule<sup>103</sup>. In view of the fact that even the condensation with malonyl-CoA ultimately leads to the incorporation of only the acetyl residue, one could well sum up the great advantages of this structural unit in biosynthesis in the term "activated acetic acid".

The important question why the fatty acid synthesis on the multienzyme complex stops at the stage of palmitic or stearic acid, no earlier and no later, is still a puzzle. On the other hand, it is easier to answer the question of the advantages that result from a synthesis of many steps taking place on a multienzyme complex. A process of this type must be kinetically far superior to analogous processes that are catalysed by separate enzymes, since the diffusion paths of the substrates are kept to a minimum by the covalent bonding to the complex. For the same reason, the local substrate concentrations in the multienzyme complex are high, so that the formation of the Michaelis complexes between the various substrates and the corresponding parts of the enzyme is also favoured. Another factor that is probably of great physiological importance is that interference in the synthesis process by foreign enzymes, such as the enzymes of the fatty acid degradation, is suppressed. In this respect, one can speak of compartmentation of biochemical reactions in a very small space. Through this spatial division of the fatty acid synthesis and the fatty acid degradation, the cell can allow both processes to take place separately and, what is more important, can control them independently. This is a great advantage, since both processes pass through the same intermediates, apart from the stereochemical difference in the  $\beta$ -hydroxy acids. The multienzyme complex of the fatty acid synthetase may be compared to the assembly workshops of industry. In both cases, the parts or components supplied from outside are fitted together and transformed piece by piece, and leave the production site only in the form of the finished product.

*Biological Regulation of the Fatty Acid Synthesis*

How is the component malonyl-CoA supplied in the fatty acid synthesis? We have already seen that it is formed by carboxylation from acetyl-CoA with the participation of a specific biotin-enzyme and ATP as the energy source (Eqn. 7). In the synthesis of fatty acids, therefore, in complete agreement with the general plan of life processes, the driving force of the synthesis process is provided by the cleavage of ATP. ATP allows the fixation of carbon dioxide to the acetyl-CoA, which is then removed again on lengthening of the carbon chain with incorporation of the  $C_2$ -unit.

The peculiarity of animal acetyl-CoA carboxylase is the need for citric acid for the development of full activity<sup>104-106</sup>. It was found on detailed investigation that the activation phenomenon falls in the field of allosteric effects<sup>107</sup>, and is connected with the aggregation of several enzyme molecules to form a polymer with a molecular weight of more than 2 million<sup>106,108</sup>. Allosteric effects are becoming increasingly important at present in explaining the regulation of metabolism in the organism. In the case of acetyl-CoA carboxylase, the citrate activation alone is evidently of minor importance. However, it becomes decisive through the fact that it is opposed by powerful antagonists in the CoA compounds of higher fatty acids such as palmitic, stearic, and oleic acids, Bortz, Numa and I<sup>108</sup> found that coenzyme A derivatives of this type inhibit the acetyl-CoA carboxylase even in very small concentration by competition with citrate<sup>108</sup>. In the presence of palmitoyl-CoA, the activation of the enzyme action and the aggregation of the protein do not take place.

These findings contribute considerably to our understanding of the biological regulation of fatty acid synthesis in the animal organism. The carboxylation reaction is the rate-limiting step<sup>109,110</sup>, and it follows that any change in the activity of the acetyl-CoA carboxylase is reflected in the rate of the fatty acid synthesis. It has been known for a long time<sup>101</sup>, that fatty acid synthesis is almost completely suppressed during starvation or in diabetes, but the fatty acid level in the blood is simultaneously increased through the mobilisation of stored fat. Even in normal animals, a high-fat diet leads to drastic inhibition of fatty acid synthesis. The concept of homeostatic control of the synthetic process that is deduced from these observations can be explained by our findings with purified acetyl-CoA carboxylase. After experiments on starved rats and on diabetic animals, the level of fatty acid CoA derivatives in the liver is considerably higher than usual<sup>111,112</sup>. As can be seen from a glance at the lipogenesis scheme (Fig. 23) this must lead to a very logical regulation of fatty acid

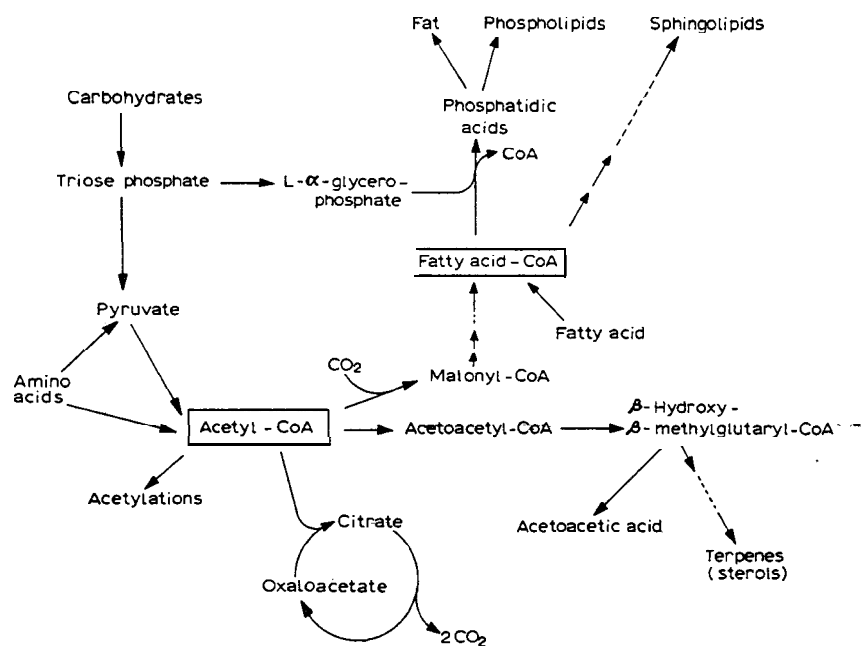


Fig. 23. Lipogenesis scheme.

synthesis. The fatty acid-coenzyme A compounds that are effective as inhibitors are the last links in the synthetic chain, from which the incorporation into the complex lipids takes place. If the fatty acid-CoA compounds accumulate in the fat-forming tissues, the very enzyme whose action causes the reaction sequence of the fatty acid synthesis to branch off from the further reactions of the "activated acetic acid", *i. e.* the acetyl-CoA carboxylase, is inhibited. In fatty acid synthesis, therefore, we again meet with the typical phenomenon of "end-product inhibition"<sup>113</sup>, whose great importance has been recognised in recent years in the study of many biosyntheses, and which makes a decisive contribution to the high economy of the processes of life. Thus an increase in the intracellular concentration of fatty acid-CoA compounds is the signal that the requirements for the synthesis of complex lipids, such as the neutral fats, the phospholipids, and the sphingolipids, have been met. In these circumstances, it would be wasteful to pass further acetyl-CoA along the path of this biosynthesis.

Inhibition of acetyl-CoA carboxylase by free fatty acids has also been reported during the past year<sup>114,115</sup>. However, these were distinctly inferior to



the corresponding coenzyme A derivatives as inhibitors in our experiments on the purified enzyme<sup>108</sup>.

The practical importance of our observations is obvious. If we could find substances that inhibit the acetyl-CoA carboxylase in the same way as the fatty acid-coenzyme A compounds, but unlike the latter are not incorporated into the neutral fats or phosphatides, it would be possible to control the fatty acid synthesis medicinally. I can see here real points of attack for directed therapy of the circulatory diseases, and this adds to the significance of the fundamental research for which the Royal Caroline Institute has awarded me the highest scientific distinction. I share this distinction with many excellent colleagues who have accompanied me on longer or shorter stretches of the road.

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