

## CURRENT THOUGHTS ON OLD PROBLEMS

P.K. Stumpf

Department of Biochemistry and Biophysics, University of California,  
Davis, California 95616, USA

Abstract -- The mechanisms of fatty acid biosynthesis have been reviewed in terms of three major breakthroughs which allowed rapid progress in the elucidation of the reactions involved in the formation of fatty acids in both procaryotic and eucaryotic organisms.

## INTRODUCTION

It has been the privilege of the author to have witnessed, participated in and partially survived the past two and a half decades of research in the field of lipid biosynthesis; in so doing, I am always struck by the inefficiency of research, not only in my own field, but in so many other phases of scientific investigation. Certainly the development of techniques, i.e., gas liquid chromatography, thin layer chromatography, continuous radio-monitoring of glc effluents as well as the availability of pure cofactors and radiochemicals at reasonable prices were major factors in the continuous advancement of knowledge in my field. But as Szent-Gyorgyi has so aptly put it "Research is to see what everyone has seen but to think what no-one else has thought", we have seen much -- just look at the many reviews on the subject -- but perhaps we have not thought as much. Suffice it to say that the search for the complete picture depicting the biosynthesis of lipids, and the regulation of these mechanisms continues at a furious pace at the present time.

I would like to trace for you some of the highlights which brought about an understanding of the mechanisms involved in the formation of fatty acids, not only in plants but also in bacterial and animal systems.

### Early History

The modern aspects of this story begin with the discovery by Lipmann (1) of coenzyme A in 1948-1949 and the elucidation of the structure of the mysterious C<sub>2</sub> unit, which was postulated as a product of  $\beta$ -oxidation and a substrate for fatty acid synthesis, as acetyl CoA by Lynen (2) in 1951.

At this point in time, two large research centers, Green's group (3) at the Institute of Enzyme Research, University of Wisconsin and Lynen's group (4) in Munich made gigantic studies in understanding the mechanism of  $\beta$ -oxidation of saturated fatty acids and by 1953 both groups announced detailed descriptions of the complete series of reactions as well as the characterization of all the enzymes involved in this process.

In the meantime, Brady and Gurin (6) in 1950 published an important paper which described for the first time the preparation of a pigeon liver homogenate that readily incorporated <sup>14</sup>C-acetate into long chain fatty acids in the presence of CoA, NADH, ATP and citrate. For a number of years thereafter, attempts were made to show that *de novo* biosynthesis was simply the reverse of  $\beta$ -oxidation but when Wakil's group persisted in showing that pigeon liver preparations could synthesize fatty acids but had no  $\beta$ -oxidation activity a realignment of pre-conceived ideas resulted.

In retrospect three milestones of discovery occurred in the brief period of about five years which revolutionized the concepts of fatty acid synthesis.

The first occurred in 1958 (6-7) when Wakil and his colleagues made the very important discovery that CO<sub>2</sub> greatly stimulated fatty acid biosynthesis in pigeon liver extracts but is not incorporated into fatty acids, that the product of CO<sub>2</sub> fixation was malonyl CoA, that malonyl CoA was very rapidly incorporated into fatty acids with the release of CO<sub>2</sub>, and that biotin was somehow involved in the carboxylation of acetyl CoA to malonyl CoA by the newly discovered enzyme, acetyl CoA carboxylase.

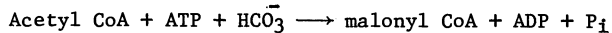
The second milestone was the discovery by Vagelos (8) and by Wakil (9) that a heat stable protein, called acyl carrier protein (ACP), was an essential component of a number of

bacterial synthesizing systems. Shortly thereafter, we showed a similar requirement in plant synthesizing systems (10).

The third milestone was the announcement by Lynen in 1961 (11) of the purification and characterization of a multi-enzyme complex in yeast which could rapidly synthesize palmityl CoA from acetyl CoA and malonyl CoA. Research is full of surprises; in 1976, a number of investigators have provided evidence revising rather significantly the multi-enzyme complex hypothesis originally proposed 13 years earlier by Lynen.

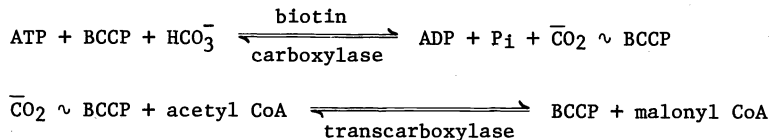
We shall now examine in more detail these three major advances and we shall attempt to interrelate these with systems described in both procaryotic and eucaryotic organisms.

Acetyl CoA carboxylase--the first milestone. Although much can be said about this enzyme (12), the final picture is just now emerging. This biotinyl enzyme catalyzes the carboxylation of acetyl CoA to malonyl CoA:



It is ubiquitous in nature. However, because of considerable variability in stability, the assay of its activity in various tissues is difficult. In fact, a better understanding of the inconsistent stability of this enzyme, particularly in plant tissues, would be a worthwhile investigation.

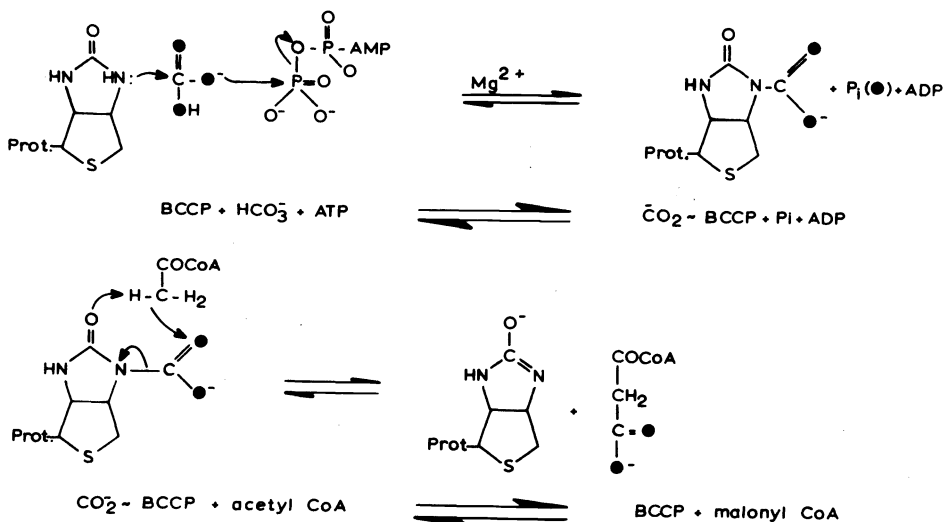
In procaryotic cells such as *E. coli* (13), the enzyme consists of three separable and distinct proteins, namely biotin carboxylase, biotin carboxyl carrier protein (BCCP) and transcarboxylase and these interact as follows:



The carboxyl group is exclusively associated with the 1'-N ureido nitrogen of the biotinyl component and has been identified by Lynen (14) as the 1'-N-carboxylmethyl biotinyl derivative. Biotin in turn is covalently bonded via a peptide linkage with the  $\epsilon$ -amino groups of a lysine residue of the specific protein, namely BCCP.

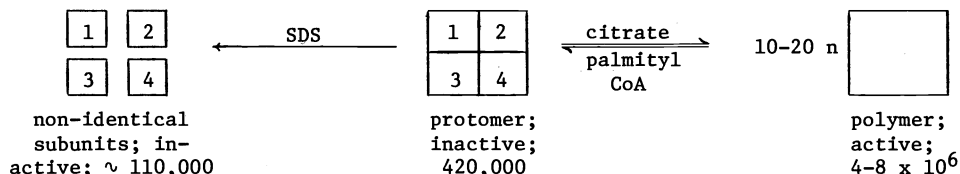
The mechanism of carboxylation is thought to be:

#### Mechanism of Carboxylation by Acetyl CoA Carboxylase



What are the molecular properties of these three discrete proteins? Biotin carboxylase, crystallized by Lane, has a molecular weight of 100,000 with two subunits of 51,000. BCCP is a single polypeptide chain with one biotin per BCCP and with a molecular weight of 22,500. It can occur as a dimer of 45,000. Transcarboxylase, the third protein, has a molecular weight of 90,000 with two subunits of 45,000 each (13).

In sharp contrast, the avian liver acetyl CoA carboxylase appears to have the following properties (12):

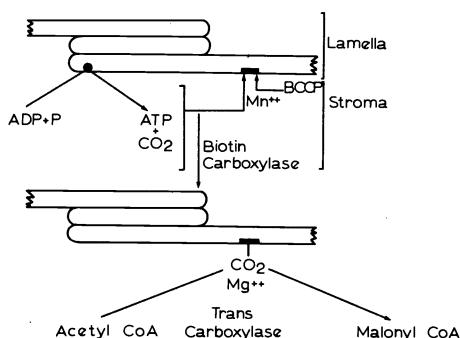


Very recently, Numa and his colleagues (15) have extensively examined the subunit structure of rat liver acetyl CoA carboxylase and have shown quite unequivocally that this protein has only one subunit with a molecular weight of 230,000 and one molecule of biotin per subunit. Earlier studies suggested several smaller non-identical subunits but Numa showed clearly that the molecular properties of the enzyme could be modified by endogenous proteases which presumably attack a region of the subunit particularly susceptible to proteolytic attack. Since this single polypeptide carries the functions of biotin carboxylase, biotin carboxyl carrier protein and transcarboxylase as well as a regulatory site, this mammalian enzyme has a highly integrated structure. We shall have more to say about multifunctional activities and single polypeptides later.

The animal acetyl CoA carboxylase is of unique importance since this enzyme catalyzes the first committed reaction in the biosynthesis of fatty acids and appears to be under two types of control, (a) allosteric regulation with citrate as the positive effector and palmityl CoA as the negative effector, and (b) adaptive changes in enzyme content based on a balance between rates of synthesis and rates of degradation, both of which are under nutritional and hormonal control. However, in sharp contrast, the acetyl CoA carboxylases in bacterial, yeast and plant tissue do not exhibit similar allosteric properties. Recently, however, we have observed that wheat germ acetyl CoA carboxylase may be activated or inactivated as a function of the  $K^+/Na^+$  function of the medium (16).

A few words should be said about a procaryotic-type acetyl CoA carboxylase in spinach chloroplasts (17). Chloroplasts have procaryotic properties in that while nuclear DNA programs much of the synthesis of chloroplastic proteins, chloroplasts do contain 70S ribosomes and their protein-synthesizing machinery displays the same inhibitory responses to some protein inhibitors as do procaryotic organisms. In 1972 we observed that the unstable acetyl CoA carboxylase of spinach chloroplasts consisted of the same three proteins as was observed with *E. coli*, but that two of the proteins, biotin carboxylase and transcarboxylase, were soluble components of the system whereas BCCP was identified as an intrinsic protein firmly imbedded in the lamellar membranes of the chloroplast. Since these membranes are the sites of photophosphorylation we have both the sites of ATP synthesis and of carboxylation, namely BCCP, closely juxtaposed for effective activation of  $HCO_3^-$  for fatty acid synthesis.

#### ACETYL CoA CARBOXYLASE IN CHLOROPLASTS

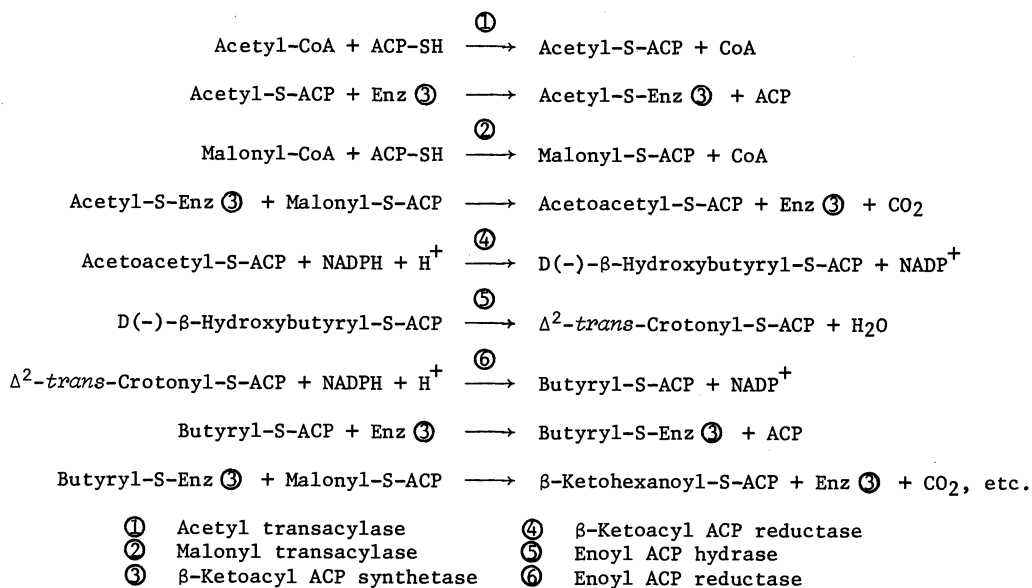


In summary, then, the present picture that emerges with acetyl CoA carboxylase is on one hand a relatively simple one in bacteria, i.e., a three soluble protein system which, in chloroplasts of higher plants, has evolved into a soluble two protein component system with

BCCP, however, membrane bound. In animal systems, however, we have an allosteric, reversible inactive protomer  $\rightleftharpoons$  active polymer system with on one hand subunit structures and on the other hand a single polypeptide system with all the activities including a regulatory site associated with four specific domains on that single polypeptide. Research in the next few years will resolve many of the problems associated with this enzyme. The results should be most interesting!

The role of ACP--the second milestone. Until about 1963 all mechanisms of fatty acid biosynthesis always involved exclusively acyl CoAs as the thioester intermediates although it was recognized that acyl groups were bound covalently to protein moieties of the synthetase enzymes. However, Vagelos and his colleagues (8) observed that in attempts to purify the bacterial enzymes involved in the synthesis of fatty acids from acetyl CoA and malonyl CoA, activity was lost but could be restored by adding a heat stable factor obtained from crude extracts and soon found to be a heat stable, acid stable, very soluble protein. Both Vagelos and colleagues and Wakil and colleagues (9) attacked this problem with great vigor and within a relatively short period of time, not only was its function defined but its complete primary structure was determined and indeed recently it has been chemically synthesized by the Merrifield procedure (18).

A few comments are in order here: (a) ACP plays a key role as the acyl carrier in many bacterial and plant synthetase systems. The following reactions involved in the *de novo* synthesis of palmitic acid in bacterial and plant systems clearly elucidate its function:



(b) Soluble ACPs have been isolated from both bacterial and plant sources (19). Whereas the amino acid residues of these proteins differ appreciably, the core amino acids surrounding the serine residue which carries the 4'-phosphorylpantetheine moiety are identical and have been conserved in the long evolutionary span between simple procaryotic organisms and complex plant tissues (20). Moreover, *E. coli* ACP functions very effectively in all plant systems so far tested although the converse, namely the inclusion of a plant ACP in a bacterial synthetase system leads to a number of interesting events. (c) Both bacterial and plant ACPs are single polypeptides with molecular weights ranging from 8,000-12,000, heat stable, acid stable at 25° and each containing one 4'-phosphopantetheine per mole of protein. *E. coli* ACP appears to be a typical globular protein, compactly folded. In the presence of high guanidine concentrations, native ACP unfolds and is reversibly denatured (21). (d) However, in some bacteria such as the advanced procaryote, *Mycobacterium phlei*, in yeast, and in *Euglena gracilis*, a polypeptide residue or domain of a larger polypeptide has a 4'-phosphorylpantetheine moiety associated with it which functions as an acyl carrier (13). (e) In all animal systems, an integrally covalently linked polypeptide with ACP properties is associated with the larger polypeptide unit.

In summary, in all *de novo* fatty acid synthesizing systems, the acyl thioester of prime importance are acyl ACPs or acyl ACP-like substrates. Acyl CoAs serve ancillary roles as primary substrates, such as acetyl CoA and malonyl CoA which are then transferred onto the polypeptides by transacylations as described previously in this chapter.

The fatty acid synthetase--the third milestone. The chemistry of the reactions from the initial condensation of acetyl thioester and malonyl thioester to the final release of the

product, palmitic acid (or thioester) is well documented (11). The interpretation of the data describing the arrangement of these enzymes in animal systems involved in these reactions has undergone fascinating changes in very recent times. A brief summary is therefore in order.

In 1961, Lynen (11) described an elegant series of experiments on the fatty acid synthetase of yeast. This study provided the first major insight into the possible mechanism which could explain the highly efficient formation of palmitoyl CoA from both acetyl CoA and malonyl CoA. The synthetase was described as a multi-enzyme complex consisting of seven different proteins held together by strong non-covalent forces. The molecular weight of this complex was 2,300,000. All of the reactions described earlier were shown to be catalyzed by this complex of enzymes, the details of which cannot be described here (13). All attempts to dissociate the complex into the seven proteins postulated to make up the complex met with varying degrees of failure. Moreover, each molecule of synthetase contained 3.5 to 6 moles of 4'-phosphorylpantetheine.

These studies greatly stimulated similar research by Vagelos and his group (13) and by Wakil and his group (22) but these workers employed the *E. coli* synthetase as a model system and they rapidly discovered, much to their delight, that unlike the yeast synthetase complex which could not be dissociated to subunits capable of catalyzing the individual steps, the *E. coli* system consisted of at least six enzymes that could be separated, purified and studied in depth. These were the acetyl CoA-ACP "transacylase," the malonyl CoA-ACP transacylase, the  $\beta$ -keto acyl ACP synthetase with acetyl CoA-ACP transferase activity, the  $\beta$ -keto acyl ACP reductase, the  $\beta$ -hydroxy acyl ACP dehydrase and the enoyl ACP reductase.

In plants, the synthetase activity cannot be sedimented by prolonged high gravitational forces and therefore it resembles the bacterial rather than the animal systems which readily sedimented under these conditions. This observation does not rule out the good possibility that the plant system may still be a single polypeptide system of molecular weight sufficiently low to remain in the supernatant rather than in the pellet after prolonged centrifugation (23).

The picture that began to emerge from 1961 to 1972 was that in all animal systems as well as in yeast, fatty acid synthetase was a tightly associated heteropolymer complex. Thus, the pigeon liver synthetase had a molecular weight of 450,000, rat liver 540,000, chicken liver 508,000, rat mammary gland 478,000, and *Mycobacterium phlei* had a huge complex of 1,700,000, and *Euglena gracilis* under heterotrophic growth conditions possessed a synthetase complex of 650,000 (13). None of these complexes could be reversibly dissociated to the units found in *E. coli* extracts.

In 1973, Schweizer and his colleagues (24) published an elegant paper in which by employing genetic analysis they clearly showed that yeast fatty acid synthetase was encoded by two polycistronic and genetically unlike gene loci, called *fas 1* and *fas 2*. Each *fas* locus encodes only a single multi-functional protein. Thus, Schweizer concluded that *fas 1* encoded for  $\beta$ -hydroxy acyl dehydrase, enoyl reductase, malonyl transferase and palmitoyl transferase while *fas 2* encoded for  $\beta$ -keto acyl reductase,  $\beta$ -keto acyl synthetase and ACP. Furthermore, the purified yeast complex could be split to two dissimilar units of 179,000 and 185,000 molecular weights, the former being the gene product of *fas 1* and the latter, the gene product of *fas 2*.

These results began to explain the long string of unsuccessful experiments by investigators attempting to dissociate multi-functional polypeptides; however, Porter and his colleagues (25) still feel that subunit structure gives the correct picture. Their pigeon liver synthetase can be dissociated into two half-molecular weight non-identical subunits with subunit I containing 4'-phosphopantetheine, eight sulfhydryl groups, and a  $\beta$ -keto acyl and crotonyl thioester reductase activity, subunit II has acetyl and malonyl transacylase activity. Both subunits have  $\beta$ -hydroxy butyryl thioester dehydrase and palmitoyl CoA deacylase activity. Of particular interest is the fact that subunits I and II can be reassociated to 75% of the original synthetase activity. Wakil and his group in 1975 (26) working with chicken liver synthetase can dissociate their complex to two dissimilar subunits of 250,000 molecular weight with loss of activity but these inactive units can be reassociated with full activity. Buckner and Kolattukudy (27) have very recently purified the fatty acid synthetase from the uropygial gland of the goose. The complex has a molecular weight of 547,000 and splits into two equal molecular weight polypeptides of 269,000 in low ionic strength or SDS. Since two moles of 4'-phosphorylpantetheine are found in the intact molecular species, the results strongly suggest two multi-functional polypeptide subunits each containing one 4'-phosphorylpantetheine moiety. Thus, the current hypothesis for the animal fatty acid synthetase suggests that the complex may consist of two polypeptides (identical or non-identical?) with multiple active sites at critical domains of the polypeptide chain rather than consisting of non-dissociating 6-7 enzyme subunits held together by extremely strong non-covalent forces. This current concept for fatty acid synthetase is strikingly similar to Numa's recent work on acetyl CoA carboxylase molecular

structure. There still remains considerable disagreement among investigators in this field but the next decade should lead to a clarification of the molecular structure(s) of the fatty acid synthetase.

#### CONCLUSION

We have considered the highlights of the events leading to the present day concepts of lipid biosynthesis both in procaryotic and eucaryotic organisms. We have suggested that progress depended on three major events which redirected the researchers in the field of lipid biosynthesis to such an extent that great and rapid advances were made. In the future, similar discoveries will be made leading to further explosive progress.

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