NEW EXPERIMENTS OF BIOTIN ENZYMES

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INTRODUCTION

In 1936 Harland Wood, as a student of Werkman, had demonstrated that, when *Propionibacterium shermanii* and other propionic acid bacteria are grown with glycerol as the substrate, there is a net uptake of CO_2. He soon (1940) also demonstrated that the amount of succinate formed is equivalent to the net uptake of CO_2, and later (1941), when $^{13}CO_2$ became available, he showed that the $^{13}CO_2$ is incorporated into the carboxyl groups of succinate. This was the first demonstration of the utilization of CO_2 in a carbon-to-carbon synthesis by any organism other than plants and autotrophic bacteria and as a most important result this discovery broke the then existing dogma that CO_2 was used only by autotrophs. Accidentally in the same year (1936), when Wood and Werkman for the first time reported the uptake of CO_2 by propionic acid bacteria, an indispensible growth factor of yeast was isolated by Fritz Kögl and his collaborators from egg yolk and was called biotin.

We now know that the fixation of CO_2 in living organisms can be achieved in various ways and in this context the carboxylation of peptide-bound glutamic acid to form γ-carboxyglutamic acid and the requirement of vitamin K as an essential cofactor is the most recent discovery.[1] However, many of the carboxylation reactions are catalyzed by biotin enzymes, as was discovered in our laboratory in studies of β-methylcrotonyl-CoA carboxylase, an enzyme which participates in the degradation of leucine by way of isovaleryl CoA.[2] This enzyme could easily be isolated from microorganisms grown on isovaleric acid as sole carbon source. The purified enzyme, being about 150 times more active than the crude extract, was found to be homogeneous as checked by sedimentation in the ultracentrifuge and by electrophoresis. By adding ammonium sulfate to the solution of the purified enzyme, we even succeeded in crystallizing the protein.[3]

It could be 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.[2,4] Subsequently, the bond between biotin and CO_2 was elucidated in the experiments of Knappe in our laboratory and shown to be 1′-*N*-carboxybiotin bound to the enzyme protein through amide linkage at the ε-amino group of a lysine residue (Figure 1).[4,5]

In recent years a controversy arose regarding the exact site on biotin to which the carboxyl group was attached. Bruice and Hegarty contended that assignment of the 1′-*N*-carboxyl as the reactive group was equivocal.[6] Their model studies indicated that the carbonyl oxygen was the most nucleophilic site on the biotin ring system and therefore they suggested that carboxylation occurred at the ureido-*O* and that, during the methylation used to isolate carboxy-biotin, *O*-carboxylated biotin underwent a chemical rearrangement that transferred the carboxyl group to the ureido-*N*. However, Lane and his colleagues have now shown in a convincing manner that 1′-*N*-carboxyl-biotin is the biologically reactive form,[7] as suggested by us in 1959.

Regarding the source of the carboxyl group bound to biotin, the biotin enzymes can be divided into two classes (Figure 2). The carboxylases utilize bicarbonate as carboxyl donor and require ATP to drive the formation of the new C–N-bond. The transcarboxylating enzymes catalyze the formation of the carboxyl-biotin-intermediate by an ATP-independent transcarboxylation with either a β-keto acid or a malonyl-CoA derivative serving as carboxyl donor.

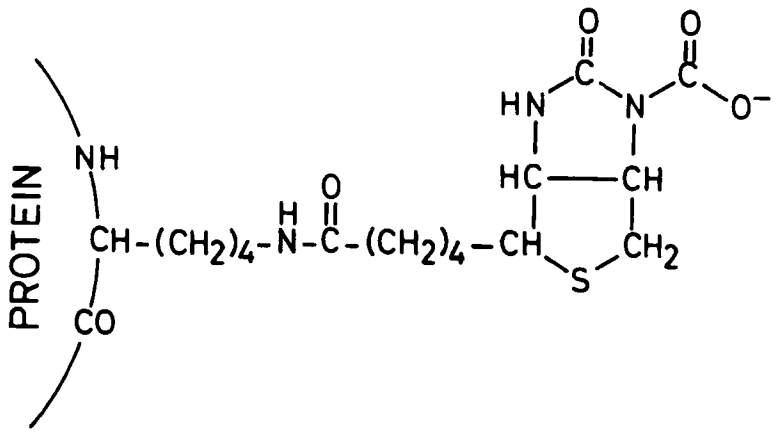

FIGURE 1. Chemical structure of carboxybiotin enzyme.

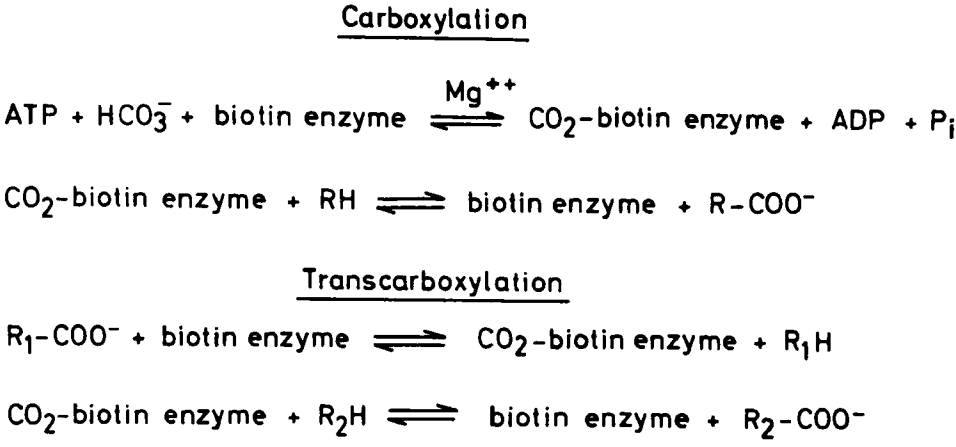

FIGURE 2. Classification of biotin enzymes.

There is now good evidence that all of the multistep reactions of the biotin enzymes can be accounted for by appropriate combinations of a few basic types of partial reactions. In their pioneering investigations, Vagelos and his group[8] as well as Wood and his colleagues[9] studying two bacterial enzymes demonstrated that biotin enzymes actually represent multienzyme systems. It was found that the biotinyl prosthetic group in acetyl-CoA carboxylase of *E. coli* and in transcarboxylase of *Propionibacteria*, respectively, resides on a small polypeptide chain, distinct from the catalytic subunits, and to function in carboxyl translocation between these subunits. As schematically demonstrated for acetyl-CoA carboxylase of *E. coli* in Figure 3, the biotin-free carboxylase component (I) catalyzes the carboxylation of the biotinyl prosthetic group on the carrier protein (CP). Following the translocation of the carboxylated biotinyl group from site I to site II, carboxyl transfer to acetyl-CoA is catalyzed by carboxyl transferase. The attachment of CO_2 to the biocytin structure seems to be very important for this function. This way the system gains a flexible arm of about 14 Å length and this makes the carboxyl translocation between the enzyme components I and II possible.

From these observations it could be concluded that the chemical reactions catalyzed by a given biotin-containing enzyme may result from the specific combination of such subunit enzymes. In realizing that ATP-dependent carboxylations of the biotinyl prosthetic group is a partial reaction common to all biotin enzymes of class I (carboxy-

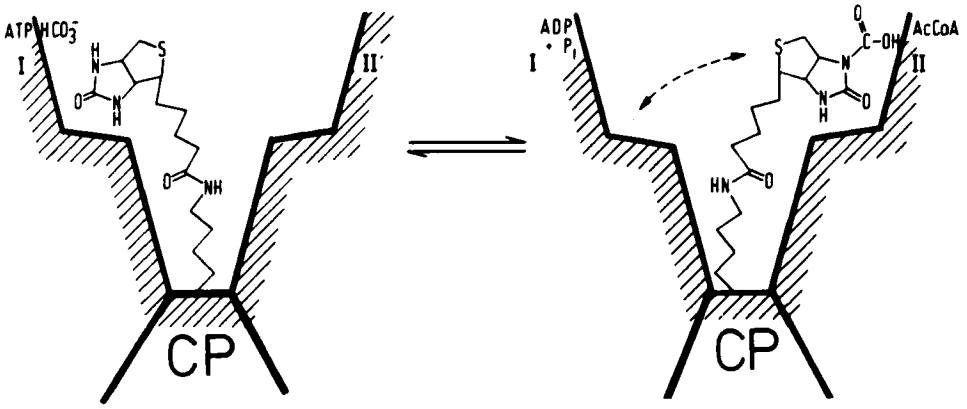

FIGURE 3. Schematic illustration of the carboxyl translocation by biotin in acetyl-CoA carboxylase of *E. coli.* (I) Carboxylase; (II) carboxyltransferase; (CP) carrier protein with its biotinyl prosthetic group.

lases), it seemed possible that for a given organism this part structure in this class of enzymes is similar or perhaps even identical. If this is true, then also the different part structures catalyzing the second step within this class of enzymes should be similar to some extent, irrespective of their different substrate specificity; at least that region of the three-dimensional structure responsible for the association with the first part structure should be similar.

STRUCTURAL STUDIES ON CARBOXYLASES OF YEAST

Pursuing this idea, Sumper[10] in our laboratory isolated the two enzymes acetyl-CoA carboxylase and pyruvate carboxylase from the same organism, namely, yeast cells, and investigated whether a structural relationship between these two enzymes exists. It turned out that by physicochemical comparison the two enzymes indeed are very similar. For example, their sedimentation coefficient was found to be identical. In addition, both carboxylases were rapidly inactivated by low ionic strength and pH values above 7.5. It could be shown that the dissociation of both enzymes (the sedimentation constant was measured to be in the native state about 16S) led to subunits with sedimentation coefficients of approximately 12, 9, and 6S, as estimated by comparison with marker proteins. In both cases it was found that subunits with sedimentation coefficients less than 15S could no longer catalyze the carboxylation of acetyl-CoA and pyruvate, respectively.

By various techniques, including the utilization of specific antibodies, prepared by injecting rabbits with the purified enzymes, it could be demonstrated that the 6S species represent the smallest units containing the complete primary structure of the native enzymes. This would mean that the heavier components of the dissociated enzymes are aggregates of different numbers of 6S subunits. Using the relationship between sedimentation coefficients and molecular weights, the sedimentation coefficients 9.5S for a dimer, 12.05 for a trimer, and 15.1S for a tetramer of the 6S subunit were obtained. These values are in good agreement with the experimentally found sedimentation coefficients. In order to find out whether the observed similarities of acetyl-CoA carboxylase and pyruvate carboxylase are also reflected in the immunochemical properties, the reaction of antiacetyl-CoA carboxylase γ-globulin with pyruvate carboxylase was studied. It was found that even a 20-fold excess of antibody, necessary to inhibit completely the acetyl-CoA carboxylase, has no effect on the pyruvate carboxylase ac-

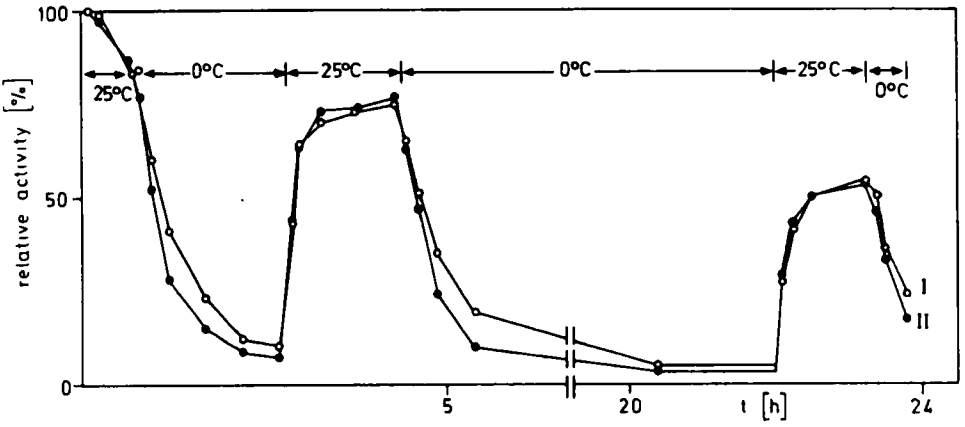

FIGURE 4. Cold lability of acetyl-CoA carboxylase of yeast. The purified enzyme, dissolved in 0.1 *M* Tris-HCl buffer, pH 7,9,10 m*M* dithiothreitol and either 60 m*M* NaCl (●) or 10 m*M* citrate (O) was kept at 25°C or 0°C, as indicated in the figure.

tivity. In a second experiment cross reaction of antiacetyl-CoA carboxylase γ-globulin with pyruvate carboxylase was checked by immunoelectrophoresis. No line of precipitation was formed however.

We thus came to the conclusion that the first partial reaction, the ATP-dependent carboxylation of biotin, common to both enzymes, is catalyzed by protein substructures which are not identical in primary structure, although having identical catalytic functions in both enzymes. On the basis of these results, we would like to assume that the genes which control the sequences of acetyl-CoA carboxylase and pyruvate carboxylase are derived from a common ancestor. By a process of one or more duplications, the ancestral gene gave rise to two or more genes which subsequently evolved independently to code for biotin enzymes with different but still similar functions.

Spiess[11] in our laboratory recently continued the studies of acetyl-CoA carboxylase of yeast. Using SDS-gel electrophoresis, he confirmed the previous finding that the enzyme represents a tetramer, built from four protomeric polypeptide chains of mol wt 190,000 daltons which probably are identical. At least protein chemical analysis revealed that the terminal amino group of all four chains is masked. On the C-terminal end Spiess found the sequence: -leu-lys.

Comparing the structures of the yeast enzyme and the *E. coli* enzyme, respectively, it becomes apparent that the three functions, located on three separable proteins in the latter are embedded in the yeast enzyme in one multifunctional polypeptide chain. In that respect the yeast enzyme resembles the mammalian enzyme, as the experiments of Numa and his colleagues[12] have shown.

The yeast enzyme differs from the mammalian enzyme insofar as it does not require citrate for activity. The addition of citrate to the solution of the purified enzyme has no effect. On the other hand, the yeast enzyme shares the cold lability with the mammalian enzyme. Figure 4 shows an experiment in which yeast acetyl-CoA carboxylase in dilute (0.1 *M*) Tris buffer, pH 8.5 of low ionic strength was incubated at 0°C. It gradually lost its enzymatic activity but regained it to a great extent when warmed to 25°C. Addition of citrate (open circles) showed practically no effect. On the other hand, the addition of 2.3×10^4 *M* acetyl-CoA protected against the cold inactivation, which probably is related to dissociation of the active oligomeric enzyme into its inactive subunits.

STRUCTURAL STUDIES ON CARBOXYLASES OF *ACHROMOBACTER IV S*

We were interested to investigate whether in other organisms the biotin containing carboxylases belong to the same category of enzymes. As an object of our studies we chose *Achromobacter IV S*, the organism from which we had isolated β-methylcrotonyl-CoA carboxylase. The structure of β-methylcrotonyl-CoA carboxylase itself was elucidated by Schiele[13] in our laboratory. When he applied the SDS-polyacrylamide gel electrophoresis technique of Weber and Osborn[14] to the pure enzyme he found only two protein bands.

By comparison with reference proteins the molecular weights were found to be 96,000 and 78,000, respectively. Assuming each polypeptide to be present in the intact enzyme, four times gives a calculated molecular weight of 700,000 which compares favorably with the value as measured by Rehn[3] with crystalline β-methylcrotonyl-CoA carboxylase in the analytical ultracentrifuge. When the radioactivity of the two polypeptides was measured, it was found that only the heavier component was radioactive, indicating that only this component carries biotin. The smaller polypeptide was free of radioactivity. Schiele succeeded in isolating the two polypeptides, called A and B, in an active state by dissociating β-methylcrotonyl-CoA carboxylase at alkaline pH and applying chromatographic techniques for the separation of the two subunits.[13] When he assayed the two proteins for enzymic activity and biotin contents, he found that protein B, the one with the larger mol wt 96,000, carries the biotin carboxylase component and the biotin carboxyl carrier protein on one and the same polypeptide chain. This protein was able to carboxylate free biotin as a substrate but was unable to carboxylate β-methylcrotonyl-CoA. Its carboxylation was only restituted when the purified protein A was also present, as illustrated in Figure 5. In this experiment the consumption of ATP linked to the carboxylation reaction was optically measured in the usual way by coupling it to pyruvate kinase and lactate dehydrogenase. As can be seen, in the presence of protein A alone in the reaction mixture, no consumption of NADH occurs. The addition of β-methylcrotonyl-CoA as substrate has no effect and only when protein B is added carboxylation of the substrate takes place. From this and many similar experiments we could conclude that protein A carries the carboxyl transferase activity of β-methylcrotonyl-CoA carboxylase.

Acetyl-CoA carboxylase from *Achromobacter* was studied by Obermayer[15] in our laboratory. He found that its structure is analogous to the structure of acetyl-CoA carboxylase of *E. coli* so extensively studied by Vagelos[19] and Lane.[20] Obermayer first purified a low molecular weight ^{14}C-biotin containing protein fraction which Schiele[16] had first observed besides ^{14}C-β-methylcrotonyl-CoA carboxylase when he grew *Achromobacter* on radioactive ^{14}C-biotin and chromatographed the crude cell extract over Sephadex G 200®. The purification procedure used by Obermayer is outlined in Figure 6.

In order to prove its identity with a specific biotin-carboxyl-carrier protein, the purified compound was coupled to the biotin carboxylase enzyme component isolated from broken *E. coli* cells according to the procedure of Lane and his colleagues.[17] In this system and in presence of ATP, Mg^{2+}, and ^{14}C-bicarbonate, it could be shown that the biotin protein became carboxylated and the CO_2 bound, as measured by the fixed radioactivity, was directly proportional to the amount of *Achromobacter* carrier added (Figure 7). For comparative reasons experiments with the purified biotin-carboxyl carrier protein of *E. coli* are included in this Figure.

In addition, Obermayer[15] could demonstrate that the carrier protein of *Achromobacter* immunologically cross-reacts with the *E. coli* carrier and can also replace it in the catalytic assay, developed by Alberts and Vagelos.[18] In this assay the carboxylation

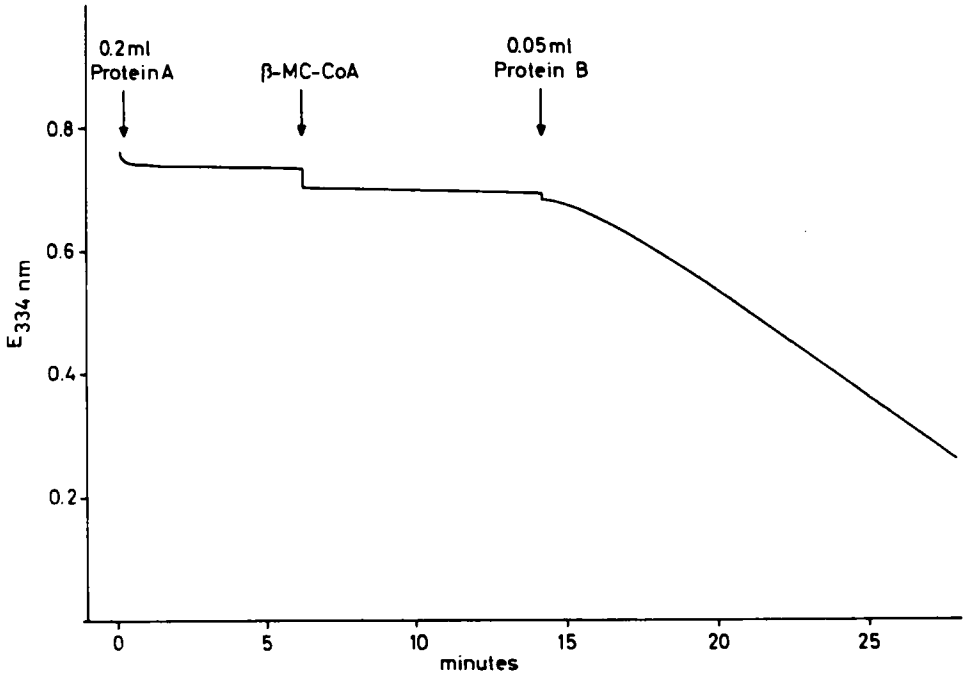

FIGURE 5. Restitution of active β-methylcrotonyl-CoA carboxylase from the separated proteins A and B. The cuvette, d = 1 cm, contained in 2 m*l*: 0.1 *M* Tris-HCl buffer, pH 8.0; 4 m*M* $MgCl_2$; 0.5 m*M* ATP; 0.75 m*M* phosphoenolpyruvate; 5 m*M* $KHCO_3$; 1.5 m*M* NADH; 1 mg serum albumin; 50 μg lactate dehydrogenase and 10 μg pyruvate kinase. λ = 334 nm, T = 25°C. At the arrows the following additions were made: 35 μg of protein A; 0.2 μmol of β-methylcrotonyl-CoA (β-MC-CoA); 4 μg of protein B.

of acetyl-CoA was measured in the presence of purified biotin carboxylase and carboxyl transferase preparations of *E. coli*. It could be further demonstrated that the *Achromobacter* carrier, as isolated by the procedure described in Figure 6, represents a degradation product of the native carrier. Similar observations have been made by Vagelos's[19] and Lane's[20] groups in their studies on the biotin carboxyl carrier protein of *E. coli*, when it was found that the protein of mol wt about 10,000, isolated at first, represented a degradation product of the native and larger carrier, the mol wt of which was found to be 22,000.

The native carrier of *Achromobacter* (mol wt 22,000) could be isolated, if the disruption of the *Achromobacter* cells, grown on acetate minimal medium containing 0.2 μ*M* ^{14}C-biotin, was performed in presence of 6 *M* guanidine hydrochloride and the protease inhibitors *o*-phenanthroline and phenylmethanesulfonyl fluoride (PMSF). The purification procedure is summarized in Figure 8.

After having identified the biotin carboxyl carrier protein of *Achromobacter*, Obermayer[15] embarked upon the isolation of the two remaining components of acetyl-CoA carboxylase from this organism. He succeeded in separating and partially purifying the biotin carboxylase component as well as the carboxyl transferase component of this organism. In these experiments it was observed that these components in the crude suspension of broken *Achromobacter* cells are in close association with the membrane fractions as isolated by sucrose density gradient centrifugation.

With the three purified components: biotin carboxylase, carboxyl transferase, and biotin carboxyl carrier protein, the acetyl-CoA carboxylase of *Achromobacter* could be reconstituted. Such an experiment is illustrated in Figure 9, where a constant

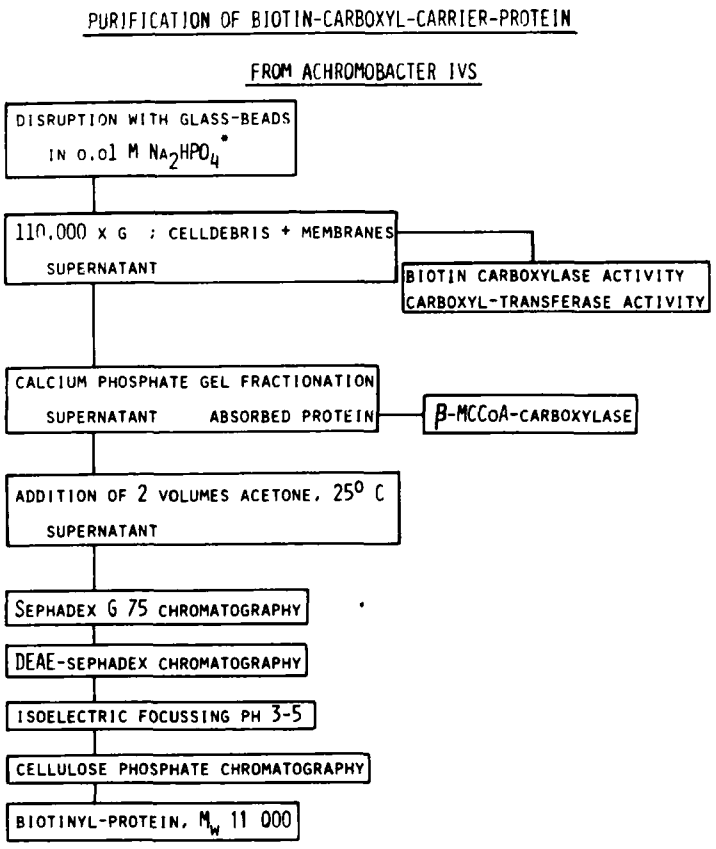

FIGURE 6. Purification of the small biotin carboxyl carrier protein from *Achromobacter IV S.*[15]

amount of biotin carboxylase and varying amounts of carboxyl transferase, as indicated on the abscissa, were incubated with 200 (I), 100 (II) and 50 (III) pmol of the purified biotin carboxyl carrier protein (mol wt 11,000) and the amount of malonyl-CoA formed by carboxylation of acetyl-CoA was measured.

In examining the family of biotin enzymes, we come to the conclusion that they can be arranged in three groups, as illustrated in Figure 10. In the first group, represented by acetyl-CoA carboxylase of *E. coli* or *Achromobacter* and the transcarboxylase of *Propionibacteria,* the active enzyme can be resolved in three types of functional components: (1) the biotin carboxyl carrier protein (C), (2) the biotin carboxylase (BC), and (3) the carboxyl transferase (CT).

In the second group, as represented by β-methylcrotonyl-CoA carboxylase from *Achromobacter,* only two types of polypeptides are present. One of them carries the biotin carboxylase activity together with the biotin carboxyl carrier protein, the other one carries the carboxyl transferase activity. Each of these polypeptides is present in the native enzyme four times. To this group of carboxylases belongs also pyruvate carboxylase of *Pseudomonas citronellolis* as the experiments of Barden and Taylor[21] have shown.

In the third group finally all three functions are incorporated in one multifunctional polypeptide chain, which again occurs several times in the native enzyme. Examples

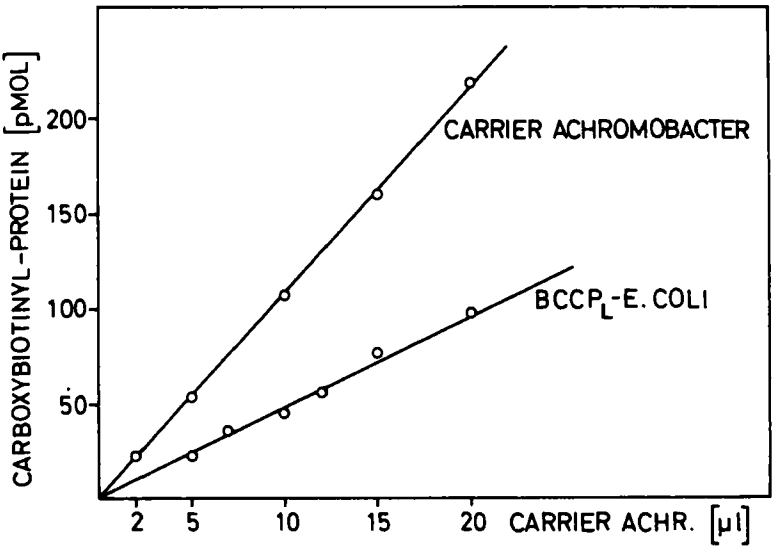

FIGURE 7. Stoichiometric carboxylation of biotin carboxyl carrier protein from *Achromobacter* and from *E. coli*. The reaction mixture contained in a volume of 50 µl: 100 m*M* triethanolamine-HCl buffer, pH 8.0; 1 m*M* ATP; 8 m*M* $MgCl_2$; 8 m*M* $NaH^{14}CO_3$ (20,000 cpm/nmol); 0.1 mU biotin carboxylase of *E. coli* and increasing amounts of biotin carboxyl carrier protein (BCCP) as indicated on the abscissa. The reaction mixture was incubated for 10 min at 30°C, then cooled to 0°C and flushed for 30 min with CO_2. After addition of NaOH the radioactivity was measured.

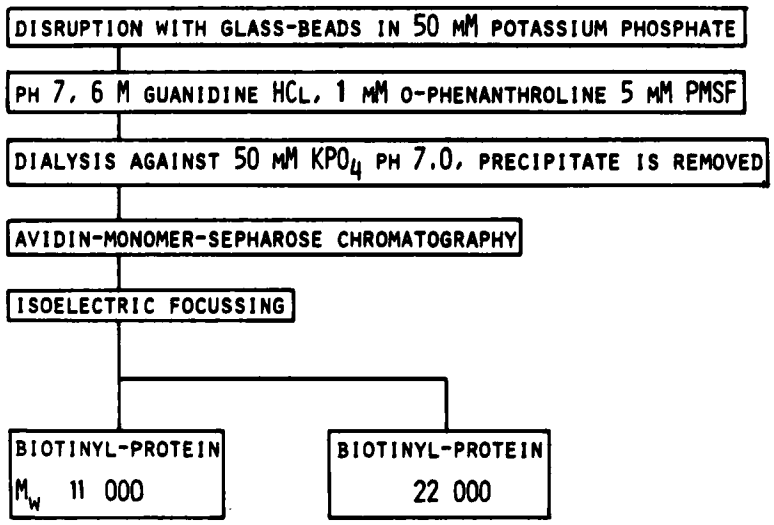

FIGURE 8. Purification of a large biotin carboxyl carrier protein from *Achromobacter IV S.*[15]

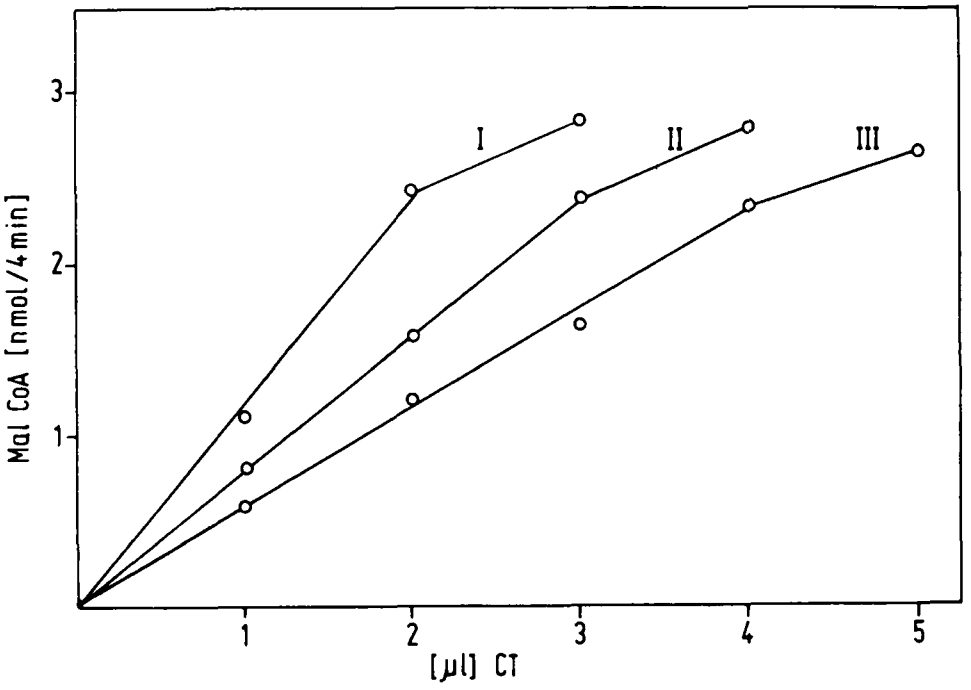

FIGURE 9. Restitution of acetyl-CoA carboxylase from its components.[15] The reaction mixture contained in a volume of 100 µl: 100 m*M* imidazol-HCl buffer, pH 8.0; 5 m*M* ATP; 8 m*M* $MgCl_2$; 2 m*M* dithiothreitol; 1.2 m*M* acetyl-CoA; 20 mU biotin carboxylase; 200 (I), 100 (II) or 50 (III) pmol biotin carboxyl carrier protein and varying amounts of carboxyl transferase as indicated on the abscissa. Incubated for 5 min at 30°C.

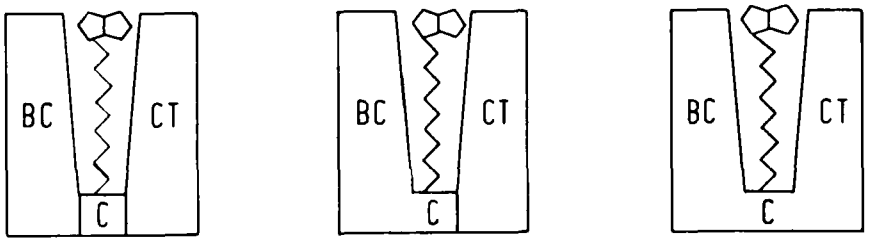

FIGURE 10. Classification of biotin enzymes according to their structures. (BC) Biotin carboxylase; (C) biotin carboxyl carrier protein; (CT) carboxyl transferase.

are acetyl-CoA carboxylase and pyruvate carboxylase of yeast as well as similar enzymes of avian and mammalian origin.[22]

It is rather attractive to speculate that the various types of carboxylases might represent various stages in the evolution of the enzyme system. According to this concept, the carboxylations may originally have been carried out by separated enzymes with an easily dissociable biotin carboxyl carrier protein. In the course of evolution the increas-

ing functional structuring of the cellular interior led to the formation of complexes with multifunctional polypeptide chains, probably as the result of gene fusion. Their biosynthesis is from the kinetic as well as the regulative point of view, superior to the formation of an aggregate composed of individual proteins. Not only the association process appears to be much simpler, but also the problem of stoichiometry with respect to the component proteins is much easier to overcome.

Our investigations, I feel, raise another interesting question. If we compare the status of *Achromobacter* with that of the yeast cell, we find that in the former the diversion of various biotin enzymes must have occurred at an early state of evolution. Otherwise the fact would be difficult to interpret that acetyl-CoA carboxylase is still on the lowest level of evolution whereas β-methylcrotonyl-CoA carboxylase is already much more advanced. On the other hand, in yeast cells the diversion between acetyl-CoA carboxylase and pyruvate carboxylase must have occurred after gene fusion had already come to an end. Otherwise it would be difficult to understand why, measured in physicochemical terms, acetyl-CoA carboxylase and pyruvate carboxylase are so similar in that organism. The answer to that question may be given by protein-chemical studies of the various enzymes, which have not yet been started but would be a great challenge for further intensive research in that field.

REGULATION OF FATTY ACID BIOSYNTHESIS AT THE LEVEL OF ACETYL-CoA CARBOXYLATION

Our studies have given special emphasis to acetyl-CoA carboxylase bcause of its role in the biosynthesis of fatty acids and its regulation.

It is well known that lipogenesis in mammals is greatly reduced in starving or diabetic animals or in animals kept on a fatty diet. In all these metabolic states the utilization of carbohydrates is reduced and the combustion of fat stands in the foreground. On the contrary, in animals given a lipid-free but carbohydrate-rich diet after starvation, lipid synthesis is greatly elevated in order to replenish the fat stores having been exhausted during the period of starvation.

When the effects of dietary conditions were studied in detail, it turned out that two types of control mechanisms have to be distinguished. One is "short term"; it can take as little as 30 to 90 min to come into effect. The other is, by comparison, "long term."

The reason for studying the level of acetyl-CoA carboxylase under various conditions is related to the fact that the first step in the biosynthetic sequence leading specifically to fatty acids is the carboxylation of acetyl-CoA. In Figure 11 all metabolic reactions forming acetyl-CoA on one side and consuming acetyl-CoA on the other are summarized. Accordingly, it would be of teleonomic significance to regulate fatty acid synthesis at the carboxylation step. In experiments with rat liver extract it was found that fasting leads to a strong depression of acetyl-CoA carboxylase and to a smaller depression of fatty acid synthetase. Similar findings were also obtained with diabetic rats as well as with rats fed on a fatty diet. On the other hand, the specific activities of both enzymes rise to very high levels in liver during high carbohydrate fat-free refeeding of starved rats.[23]

The concentration of other liver enzymes contributing to the net conversion of glucose to triglyceride are also influenced by the dietary regime. Glucose-6-phosphate-dehydrogenase, citrate cleavage enzyme and malic enzyme are depressed in starvation and become elevated in response to refeeding. Citrate cleavage enzyme catalyzes the ATP-dependent cleavage of citrate into acetyl-CoA and oxaloacetate.

$$\text{ATP} + \text{CoA} + \text{citrate} \longrightarrow \text{acetyl} - \text{CoA} + \text{oxaloacetate} + \text{ADP} + P_i$$

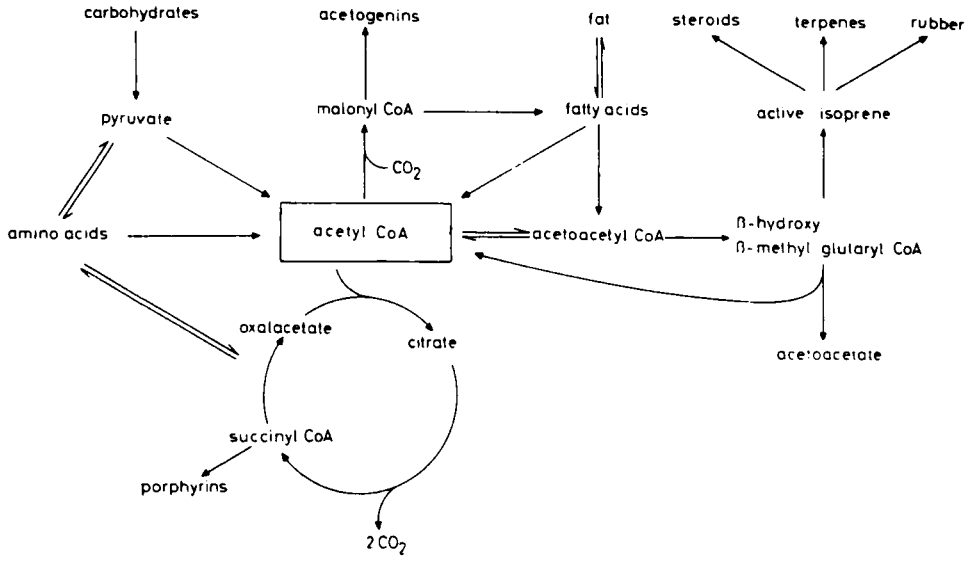

FIGURE 11. Metabolic pathways of acetyl-CoA.

There is convincing experimental evidence that this citrate cleavage enzyme is involved in the transport of acetyl-CoA formed from pyruvate inside the mitochondria into the extramitochondrial compartment of the cell where the enzymes for fatty acid synthesis are located.[24,25]

Another enzyme which may be involved in fatty acid biosynthesis is the malic enzyme, catalyzing the following reaction:

$$\text{Malate} + \text{NADP}^+ \leftrightharpoons \text{Pyruvate} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

As Ball[26] suggested, this enzyme is related to the production of NADPH required for fatty acid synthesis. According to this concept, oxaloacetate, produced by the cleavage of citrate, is reduced by NADH, formed during the conversion of glucose to pyruvate. The malate produced is then oxidized by malic enzyme to pyruvate and carbon dioxide with the generation of NADPH. The pyruvate then enters the mitochondria and there it is carboxylated to form oxaloacetate, in the presence of pyruvate carboxylase and ATP.

$$\text{Pyruvate} + \text{bicarbonate} + \text{ATP} \longrightarrow \text{oxaloacetate} + \text{ADP} + \text{P}_i$$

It was suggested that the depression of enzyme activities during starvation or in the diabetic state is due to the repression of enzyme synthesis. Evidence supporting this view has emerged in a number of studies, in particular those of Numa[27] and of Majerus.[28] These authors carried out combined immunochemical and isotopic studies with normally fed, fasted, and fat-free refed rats as well as alloxan-diabetic rats and measured in each case the rates of enzyme synthesis and of enzyme degradation, respectively. On the basis of their experimental results, as summarized in Table 1, it is evident that the rate constant of carboxylase degradation is essentially the same in normally fed, refed, and alloxan-diabetic rats, and that the increase or decrease in the carboxylase content can be attributed to accelerated or retarded synthesis of the enzyme. The values for the enzyme content predicted from the ratio of rates: k_s/k_D agree fairly well with the values actually found. The accelerated enzyme degradation observed in fasted

TABLE 1

Content, Synthesis, and Degradation of Liver Acetyl-CoA Carboxylase under Different Metabolic Conditions

Condition	Enzyme content (E)*	Rate of synthesis (K_s)	Rate of degradation (K_D)	K_s/K_D
	%	%	%	%
Rats				
Normal	100	100	100	100
Fasted	28	54	190	28
Refed	376	405	107	378
Diabetic	53	59	100	59
Mice				
Normal	100	100	100	100
Obese	1020	775	58	1340

* In steady state, $E = K_s/K_D$

rats may be due to the fact that animals during fasting are not in a steady state. In recent years Numa and his colleagues[29] could demonstrate that the regulation of enzyme synthesis occurs at the transcriptional step. They identified specific polysomes involved in the synthesis of acetyl-CoA carboxylase and found the contents of these polysomes in the livers of animals in various metabolic conditions in good agreement with the relative rate of synthesis of acetyl-CoA carboxylase.

It was found, however, that the rate of fatty acid synthesis is regulated not only by changes in the quantity of acetyl-CoA carboxylase but also by changes in the catalytic efficiency of the enzyme.[23] One of the unique features of this enzyme from animal sources is its activation by tri- and dicarboxylic acids, of which citrate and isocitrate are by far the most effective. It was discovered by Vagelos,[30] and confirmed by the studies of Numa[31] in our laboratory and of Lane,[32] that this activation by citrate or isocitrate, affecting the v_{max} of the enzyme, is accompanied by the aggregation of the inactive protomeric form of the enzyme to an active polymeric form. Further insight into the allosteric regulation of acetyl-CoA carboxylase was gained when Bortz[33] in our laboratory discovered the enzyme inhibition by long-chain acyl-CoA derivatives. Numa[34,35] carried out systematic kinetic studies with purified acetyl-CoA carboxylase from rat liver and found the inhibition with palmityl-CoA or stearyl-CoA to be competitive with regard to citrate, the activating effector of the enzyme, but noncompetitive with regard to the substrates acetyl-CoA, bicarbonate, or ATP.

We believe that these findings represent part of the cellular control mechanism. Elevated concentrations of fatty acids in the blood as a result of increased lipolysis in adipose tissue are associated with starvation, diabetes, or alimentary fat-loading, conditions in which fatty acid synthesis is known to be almost fully blocked. An elevated concentration of fatty acids in the blood may lead to an influx of fatty acids into the tissues where they become esterified with coenzyme A. In agreement with this, it was found in several laboratories[23] that the concentration of the long chain acyl-CoA compounds in rat liver is markedly increased under all conditions of depressed fatty acid synthesis.

We thus came to the conclusion that the "short term" control of fatty acid synthesis also affects acetyl-CoA carboxylase. In order to further prove the concept represented, we recently embarked on direct measurements of the level of malonyl-CoA in the livers of animals under different metabolic conditions. On the basis of our concept in all states of depressed fatty acid synthesis the level of malonyl-CoA was to be expected

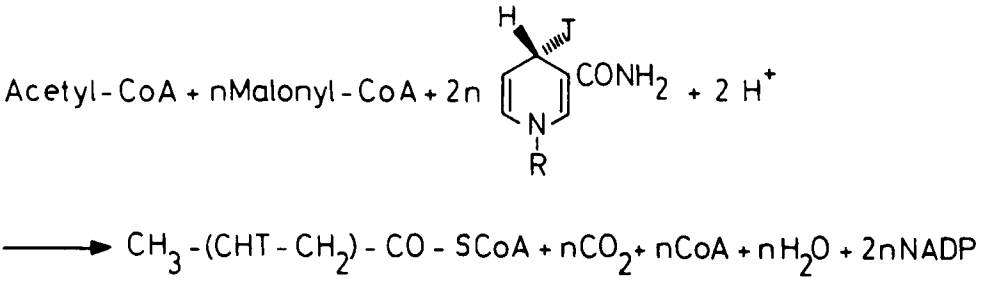

FIGURE 12. Incorporation of tritium from NADP^{3}H into fatty acids.

much lower than under normal conditions because the carboxylation of acetyl-CoA is strongly reduced and thus cannot keep pace with the consumption of malonyl-CoA by fatty acid synthetase.

As a first step, Foerster[36] developed a method for the determination of malonyl-CoA in tissues. A prerequisite for this method was a high sensitivity because the levels of malonyl-CoA were expected to be rather low. This problem could be solved in the following manner. The protein-free tissue extract was incubated with tritiated NADPH in the presence of pure fatty acid synthetase of yeast with the result that the malonyl-CoA present was quantitatively converted into radioactive fatty acids which could be extracted with petrol ether and their radioactivity measured. Applying this method it was suitable to use NADPH labeled stereospecifically at its B-side with tritium because it is known that fatty acid synthetase transfers hydride ions from this side in the two reductive steps.

According to the equation shown in Figure 12 only one tritium atom became incorporated into fatty acids per molecule of malonyl-CoA. This can be explained by the fact that the reduction of the β-keto acid intermediates involves the direct transfer of the hydride ion from NADPH to the substrate, whereas the second reduction, namely, the reduction of the double bond of the unsaturated fatty acid intermediate, occurs with flavine monophosphate as a hydrogen carrier. In the leucoflavine formed intermediately the hydrogen atoms bound to nitrogen could rapidly exchange with the protons of water. Whether this explanation is correct should be investigated by further experiments.

In his assay Foerster used highly labeled NADPH prepared by reduction of NADP with 1-^{3}H-glucose-6-phosphate in presence of glucose-6-phosphate dehydrogenase. Using this tritiated NADP^{3}H the calibration curve followed a straight line even at low malonyl-CoA concentration, as shown in Figure 13. The high sensitivity of this method is indicated by the fact that as little as 10 pmol of malonyl-CoA can still be measured accurately.

When the assay procedure was applied to tissue extracts a second problem arose. In these extracts not only malonyl-CoA but also acetyl-CoA is present and the latter may disturb the measurements. Especially then, if extracts are compared with different levels of acetyl-CoA. In order to overcome this problem, we first added large amounts of acetyl-CoA to our assay-mixture with the idea eventual differences in the acetyl-CoA concentration to overcompensate by the large amounts added. However, this method had another disadvantageous effect. The 15- to 200-fold excess of acetyl-CoA relative to malonyl-CoA shifts fatty acid synthesis to the side of short chain fatty acids as was already known from previous studies of Sumper and Oesterhelt[37] in our laboratory. If the concentration of acetyl-CoA surpasses that of malonyl-CoA by a factor of 200, more than 50% synthesized acids were present as hexanoic acid, with the result that a large amount of radioactively labeled short chain carboxylic acids remains in the aqueous phase extracted with petrol ether.

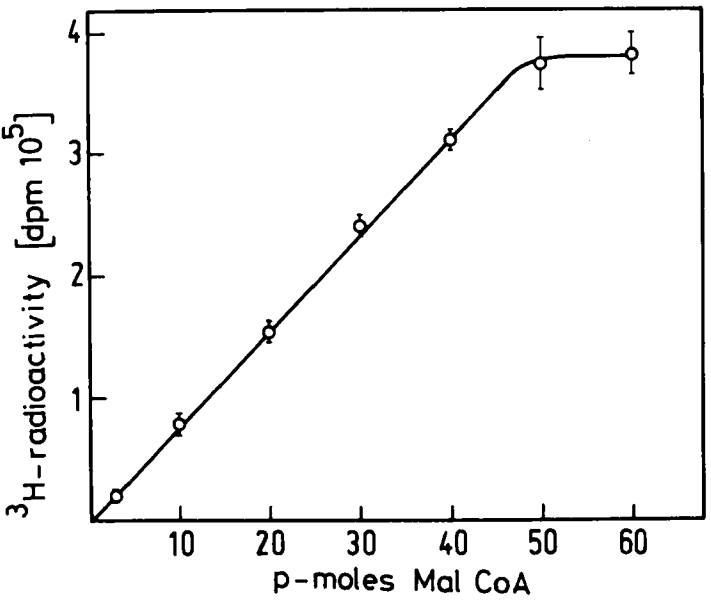

FIGURE 13. Radioactive assay of malonyl-CoA.

In the assay for malonyl-CoA, it thus became necessary to use a constant and small amount of acetyl-CoA, and that could only be achieved by the removal of acetyl-CoA present in the tissue extract. For that purpose the tissue extract was preincubated with excess oxaloacetate in presence of citrate synthase, then heated in order to inactivate the enzyme, and after addition of a small and constant amount (10 pmol) of acetyl-CoA used for the determination of malonyl-CoA.

The new assay was first applied in comparative studies of the malonyl-CoA levels in the livers of fed animals, of starved animals, and of fed animals after starvation (Figure 14). As can be seen in the figure, the normal malonyl-CoA concentration which amounts to about 7 nmol/pg of wet liver drops to less than 10% within a starvation period of 24 hr and even somewhat further if the starvation period is extended to 48 hr. After 48 hr starvation feeding restitutes the normal malonyl-CoA level.

A strong decrease in the malonyl-CoA concentration is also found in the alloxan-diabetic rat, concomitant with the increase of blood-glucose (Figure 15). In these experiments the alloxan-treated rats were first substituted with insulin, which compared with normal animals led to an increase in malonyl-CoA concentration and a concomitant decrease of glucose concentration in the blood. Withdrawal of insulin 24 hr before killing the animal induced the diabetic state.

The effect of feeding different diets to starved rats was also studied by Foerster. The results are summarized in Figure 16, where the duration of refeeding is indicated on the abscissa and the malonyl-CoA concentration found after various times is indicated on the ordinate. As can be seen in this figure, feeding a fatty diet (either the rind of bacon or a mixture of palmitic acid and casein) kept the malonyl-CoA level down. On the other hand, as the result of feeding a carbohydrate-rich diet, such as rusk or a mixture rich in starch and saccharose, the malonyl-CoA levels in the initial feeding period even surpassed the levels found after feeding a balanced diet. It is interesting to note that these high malonyl-CoA concentrations leveled down after 12 to 24 hr which may be related to an increase in fatty acid synthetase activity induced by the carbohydrate-rich diet. The results of feeding a pure protein diet (albumin) is also recorded in Figure 16. In this case the increase in malonyl-CoA concentration is much less pronounced and remarkably is followed by a strong decrease after 24 hr.

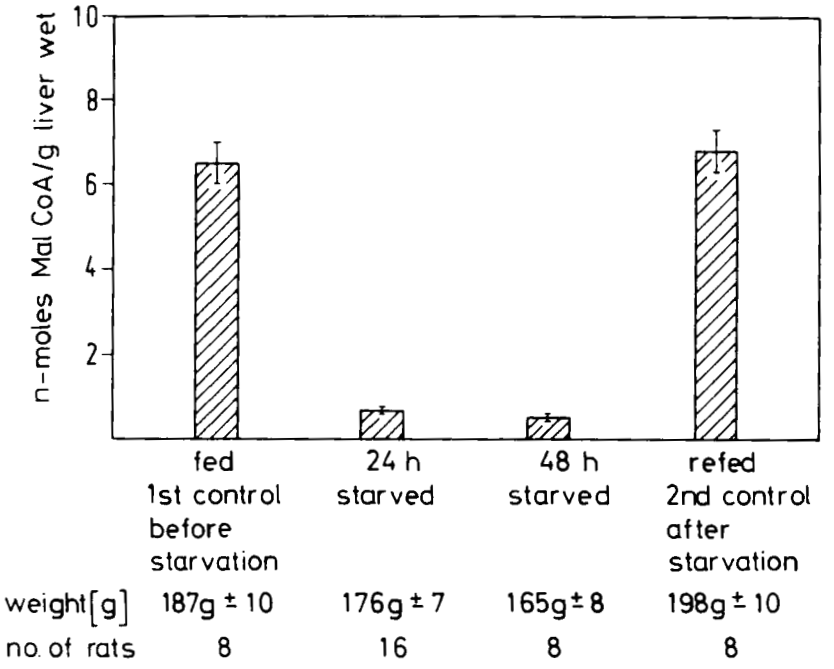

FIGURE 14. Effect of starvation on the malonyl-CoA content of rat liver.

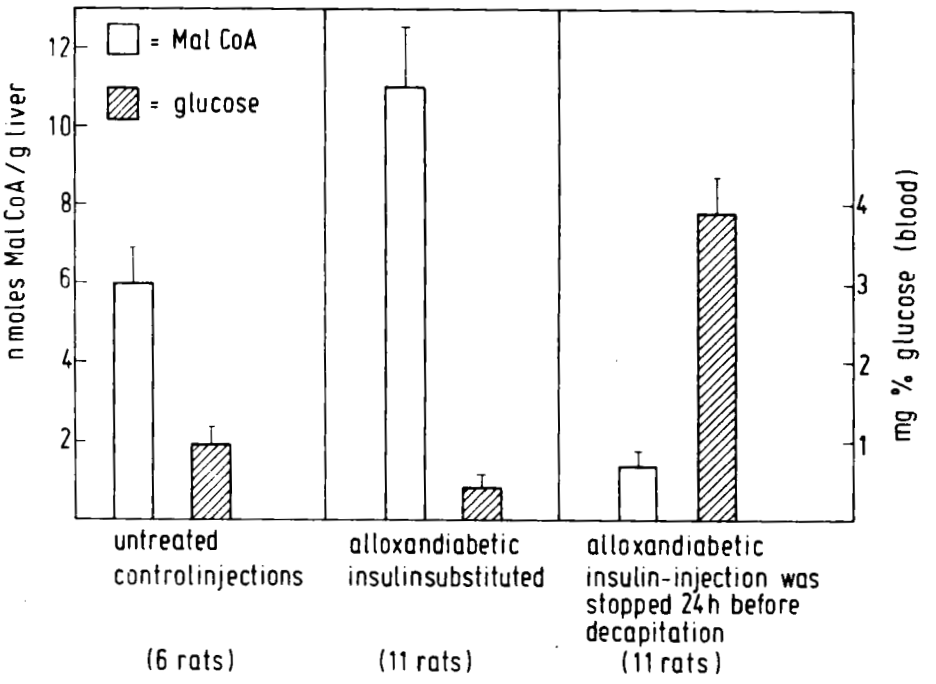

FIGURE 15. Malonyl-CoA concentration in rat livers of diabetic and insulin-substituted animals.

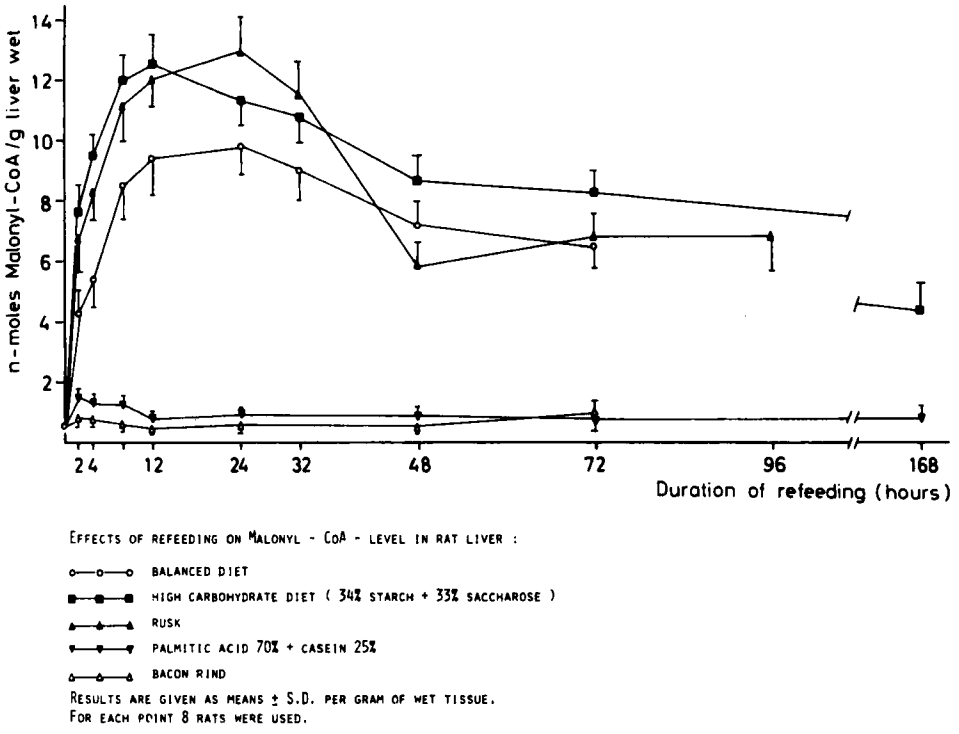

FIGURE 16. Effect of feeding different diets to starved rats.

Surveying the results of Foerster's experiments, we find our expectations realized. In all states of depressed fatty acid synthesis, such as induced by starvation, by diabetes, or by fat feeding, the level of malonyl-CoA was very low, and this is in perfect agreement with the concept that fatty acid synthesis is principally regulated at the acetyl-CoA carboxylase reaction.

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