

# Inhibition of Acetyl CoA Carboxylase by a High Molecular Weight Protein in Rat Liver

Mostafa N. Abdel-Halim\* and S. Y. K. Yousufzai\*\*

Department of Physiological Chemistry University of Wisconsin, Madison, Wisconsin 53706 USA

Z. Naturforsch. 37 c, 308 – 313 (1982); received April 29/November 2, 1981

Acetyl CoA Carboxylase, Protein Inhibitor, Rat Liver

A high molecular weight protein (approximately  $1.5-2 \times 10^6$  daltons) has been found in rat liver cytosol to inhibit acetyl CoA carboxylase activity. The protein inhibitor was purified by ammonium sulfate precipitation, DEAE-cellulose chromatography and gel filtration. The inactivation of the carboxylase is not attributable to either phosphorylation of the enzyme or to action on substrates or cofactors of the reaction. The activity of the inhibitor is destroyed by heating to  $55^\circ\text{C}$  for 5 min, by treatment with trypsin, or by increasing bovine serum albumin in the reaction mixture. Hence it appears that the inhibitor is a regulatory protein that acts directly on acetyl CoA carboxylase.

## Introduction

Acetyl CoA carboxylase (EC. 6.4.1.2.) is the rate-limiting enzyme in catalyzing the first step in a series of reactions leading to the synthesis of long chain fatty acids from acetyl CoA [1]. Several studies have proposed different mechanisms on regulation of this enzyme. Some reports indicated that acetyl CoA carboxylase is regulated via phosphorylation (inactive) and dephosphorylation (active enzyme) mechanism [2–6]. Other studies suggested that the concentration of allosteric effector such as citrate increases enzyme activity *in vitro* while palmitoyl CoA decreases enzyme activity [7]. In our earlier reports we proposed a new mechanism by which acetyl CoA carboxylase may be regulated by an endogenous protein at the *in vitro* [8–10] and *in vivo* [11] levels. In this communication, we are first to report another endogenous protein from rat liver cytosol which inactivates acetyl CoA carboxylase. The isolation, purification and some characteristics of this high molecular weight protein are the topic of this study.

## Materials and Methods

### Purification of protein inhibitor

Male Holtzman rats (Holtzman Co., Madison, Wisconsin) weighed about 180–200 g were fasted

\* Department of Human Oncology, Madison, Wisconsin 53792.

\*\* Department of Medicine, Madison, Wisconsin 53706.

Reprint requests to Dr. M. N. Abdel-Halim, Department of Human Oncology, 600 Highland Ave. K4/319 Madison, Wisconsin 53792, USA.

0341-0382/82/0300-0308 \$ 01.30/0

48 h prior to killing. The livers were homogenized and centrifuged at  $14\,000 \times g$  for 20 min. The supernatant was further centrifuged at  $100\,000 \times g$  for 75 min. The supernatant of high speed centrifugation was then adjusted to 30% with saturated ammonium sulfate; the resulting supernatant was further adjusted to 40% with respect to ammonium sulfate. The resulting pellet was then suspended into a pH 7.5 buffer containing 10 mM potassium phosphate, 10 mM potassium citrate, 1 mM EDTA, 5 mM B-mercaptoethanol and 10% glycerol (buffer A). The protein inhibitor suspension was dialyzed in buffer A at  $4^\circ\text{C}$  overnight. One hundred milliliters (800 mg of protein) of dialyzed solution was applied on DEAE cellulose column ( $3.5 \times 25$  cm bed volume). The column was initially eluted with buffer A until 280 nm absorbing material was eluted. The remaining protein was eluted with a linear phosphate buffer gradient in a range of 10 mM to 0.7 M. Flow rate was 4 ml/min and 9 ml fractions were collected. The bound protein to DEAE-cellulose was eluted by high concentration of phosphate buffer (around 0.25 M) was found to inhibit acetyl CoA carboxylase activity. The inhibitory fractions were pooled and concentrated with ammonium sulfate and followed by dialysis for 15 h at  $4^\circ\text{C}$ . Five milliliters (35 mg of protein) of dialyzed fraction was applied on Biogel A 1.5 m column ( $3 \times 55$  cm bed volume). The protein was eluted at  $4^\circ\text{C}$  with a pH 7.5 buffer containing 10 mM potassium phosphate, 5 mM potassium citrate, 1 mM EDTA, 1 mM DTT (buffer B) and 10% glycerol at a flow rate 0.25 ml/min. Four milliliters fractions were collected. The fractions which inhibited the enzyme activity were pooled, con-

centrated with ammonium sulfate and dialyzed before rechromatographed on Biogel A 1.5 m column. The fractions which inhibited 50% or more of acetyl CoA carboxylase activity were pooled again, concentrated with saturated ammonium sulfate and dialyzed.

#### Preparation of acetyl CoA carboxylase

Liver acetyl CoA carboxylase was prepared from rats fasted for 48 h followed by feeding fat free diet for 48 h prior to killing. Purification of enzyme was achieved by ammonium sulfate precipitation, DEAE-cellulose chromatography and gel filtration on sepharose 4B which replaced the linear sucrose gradient as reported earlier [8].

#### Enzyme assay

Enzyme activity in absence of protein inhibitor was assayed by measuring  $^{14}\text{C}$  incorporation into malonyl-CoA as described by Majerus *et al.* [12]. Inactivation of the enzyme by the protein inhibitor was the same as described earlier [8].

#### Gel electrophoresis

The purity of protein inhibitor was examined by gel electrophoresis. The non-denaturing gel was performed with 7.5% resolving gel (pH 8.9) and 2.5% staking gel (pH 7.2) as described by Ornstein and Davis [13]. The length of resolving and staking gels

were 7.5 and 2.5 cm respectively, with a gel tube inner diameter of 0.55 cm. In order to determine that the protein band is coincided with the inhibitory activity, the gel slices (2 mm) were soaked in buffer B overnight at 4 °C. SDS-polyacrylamide gel electrophoresis was done according to the procedure of Laemmli [14]. The gel system containing 0.1% SDS consisted of a 7.5% polyacrylamide resolving gel (pH 8.9) and 2.5% staking gel (pH 7.2). The dimensions of the gels were the same as above. The purified protein inhibitor was treated with 0.1% SDS and heated at 100 °C for 3 min.

#### Protein measurement

Protein was measured by the method of Lowry *et al.*, with bovine serum albumin as standard [15].

### Results

#### Purification of protein inhibitor

The absorbance and the percentage of inhibitor of the fraction from DEAE-cellulose chromatography is shown in Fig. 1. The anion exchange chromatography resulted into two protein inhibitors. The major activity was unbound to the resin. Detailed studies on that inhibitor was reported earlier [8]. The protein bound to the resin was eluted with higher concentration of phosphate-buffer, concentrated with ammonium sulfate, dialyzed and chromatographed on Biogel A 1.5 m column (Fig. 2). When the protein

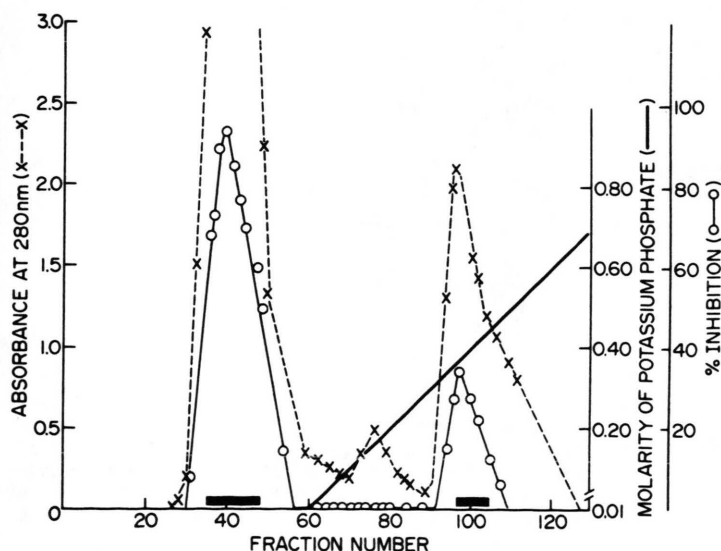
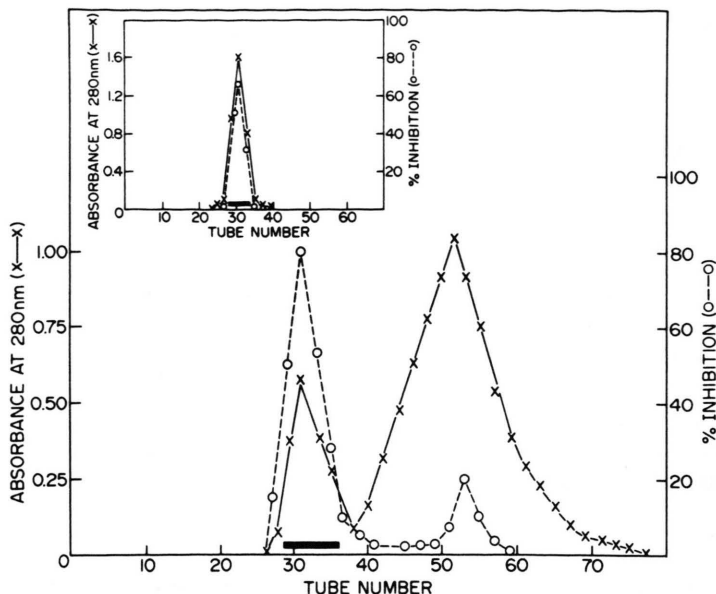


Fig. 1. Anion exchange chromatography. One hundred milliliters (800 mg of protein) of the dialyzed solution of the 30–40% ammonium sulfate was applied on a DEAE-cellulose column (3.5 × 25 cm bed volume). Flow rate was 4 ml/min and 9 ml fractions were collected. Details of this study was described in the Materials and Methods Section.

Fig. 2. Gel filtration. Five milliliters (35 mg of protein) of the DEAE-purified inhibitor was applied on a Biogel A 1.5 m column (3 × 55 cm bed volume). Flow rate was 0.25 ml/min and 4 ml fractions were collected. The inset represents the rechromatographing of the material obtained from the first run after concentrated with ammonium sulfate and dialysis, under the same conditions which was applied in the first run.



inhibitor was rechromatographed on Biogel A 1.5 m, the protein peak was very well coincided with the inhibitory activity (inset of Fig. 2). The elution of the inhibitor immediately after void volume revealed the molecular weight of inhibitor is over  $1.5 \times 10^6$  daltons. Fig. 3 shows the purity of inhibitor on non-denaturing gel and SDS gel electrophoresis. A major single band was observed on non-denaturing and SDS gel electrophoresis. The non-denaturing electrophoresis indicated that the protein band is well coincided with the inhibitory activity of the enzyme. An approximate of 80% and 75% of protein and its inhibitory activity was recovered from the major band, respectively.

It should be pointed out that an approximate of 0.2 mg of protein inhibitor was obtained from one gram liver.

#### *Inactivation of acetyl CoA carboxylase*

When highly purified enzyme (7.5 units of activity/mg of protein) was incubated with different concentrations of the purified inhibitor, the percent of inhibition was increased as the concentration of inhibition increases (Fig. 4). Therefore, the inactivation of the carboxylase by the inhibitor was found concentration dependent.

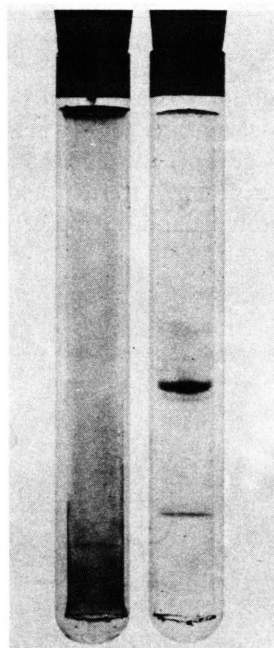


Fig. 3. Gel electrophoresis. Fifty microgram of the purified protein inhibitor was applied on polyacrylamide gels in absence (left) and presence (right) of 0.1% SDS. Electrophoresis was carried out at 5 ma per tube for 3 h in presence of a tris-glycine buffer of concentration 0.025 and 0.25 M respectively. Bromophenol blue was used as a marker and the gels were stained with coomassie blue.

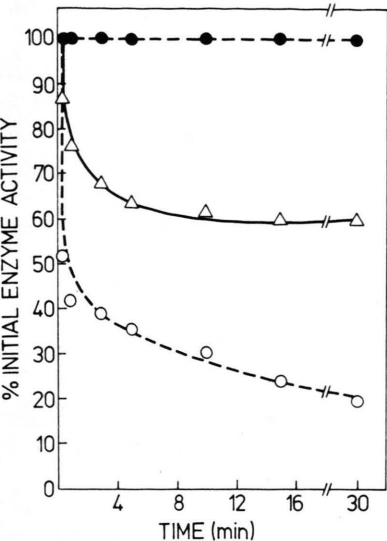


Fig. 4. Enzyme inactivation as function of time and concentration. Rat liver acetyl CoA carboxylase (specific activity 7.5 units per mg protein) was incubated with pH 7.5 solution which contained 20 mM citrate, 30 mM Tris, 4 mM MgCl<sub>2</sub> and 1 mM DTT at 37 °C for 5 min. A mixture of 0.1 mM acetyl CoA, 10 mM sodium carbonate, 2 mM ATP and 1 μCi [<sup>14</sup>C]bicarbonate was added simultaneously with either 100 μg (Δ-Δ) or 200 μg (○-○) of the protein inhibitor. The total volume of the assay was 0.5 ml. The reaction was terminated after either 1/2, 1, 3, 5, 15, or 30 min, by 0.2 ml of 5 N HCl. The mixture was evaporated to dryness in a water bath adjusted at 80 °C. The residue was dissolved into 0.5 ml distilled water and counted for radioactivity in the presence of Toluene scintillation liquid. The percent of initial enzyme activity was calculated with respect to the enzyme without the protein inhibitor (●-●).

Enzyme inactivation as function of time

It has been reported that inactivation of acetyl CoA carboxylase by protein kinase fraction is time dependent [16, 17]. The present study was performed to examine whether the protein inhibitor behaves in the same pattern or not, the enzyme and Mg-ATP were incubated with the protein inhibitor at different intervals of time. The inactivation of enzyme was reached maximum after 5 min (Fig. 4). In contrast to these results Lee and Kim [16] reported an inactivation of 83% of acetyl CoA carboxylase activity by ATP in presence of protein kinase required 90 min, however, Hardie and Cohen [17] reported that 0.3 mol of phosphate incorporated/subunit in 3 h. Therefore it may be suggested that protein inhibition is not protein kinase and hence the inactivation of acetyl CoA carboxylase is not regulated by enzyme phosphorylation.

Effect of inhibitor on the substrate and cofactors of the reaction mixture

When the inhibitor was heated alone in a water-bath for 5 min at 50 to 55 °C its activity was completely abolished. Based on this finding experiments were carried out to determine whether the inhibitor had any effect on any of the components of the incubation mixture other than acetyl CoA carboxylase prior to heating at this temperature. An aliquot of either inhibitor or deionized water (to serve as a control) was incubated separately for 30 min at 37 °C with each component of reaction mixture at the concentration used in the assay procedure. Three sets of tubes were used for this study, and each set was heated for 5 min at 45 or 55 °C. The tubes were cooled to 37 °C prior to enzyme assay. Under these conditions the inhibitor had no effect on any of the components of reaction mixture other than acetyl CoA carboxylase (Table I). Since preincubation of the inhibitor with cofactors or substrates did not alter the magnitude of carboxylase activity, hence it is concluded that inhibitor has a direct effect on the enzyme.

Effect of trypsin on the inhibitor activity

Trypsin (10:1 w/w) was incubated with the inhibitor in presence of 5 mM CaCl<sub>2</sub> at pH 8 and 37 °C, for 15 or 45 min. Trypsinization was terminated by adding soya bean trypsin inhibitor, and the activity of the inhibitor on acetyl CoA carboxylase was investigated. Acetyl CoA carboxylase incubated

Table I. Effect of inhibitor on the components of the reaction mixture. Each of the components in the incubation mixture was incubated separately without and with 200 μg of the protein inhibitor for 30 min at 37 °C. Each mixture was then heated at 45 or 55 °C for 5 min and cooled to 37 °C prior to assay for acetyl CoA carboxylase activity. In the control sample, 200 μg of the protein inhibitor decrease the formation of product by acetyl CoA carboxylase (specific activity 7.5 Units per mg protein) from 10 nmol to 2.5 nmol/min.

| Reactant                      | % Inhibition at different temperatures |       |       |
|-------------------------------|----------------------------------------|-------|-------|
|                               | 37 °C                                  | 45 °C | 55 °C |
| Citrate                       | 75                                     | 25    | 0     |
| MgCl <sub>2</sub>             | 75                                     | 26    | 0     |
| Acetyl CoA                    | 75                                     | 27    | 0     |
| HCO <sub>3</sub> <sup>-</sup> | 75                                     | 25    | 0     |
| ATP                           | 75                                     | 24    | 0     |

Table II. Effect of different concentrations of bovine serum albumin on acetyl CoA carboxylase inhibition. Assays for acetyl CoA carboxylase were carried out at different concentrations of bovine serum albumin in presence and absence of the inhibitor (200 µg of protein). Details of the assay and determination of acetyl CoA carboxylase was described earlier [8].

| Albumin Concentration [mg] | Acetyl CoA Carboxylase activity [nmol/min] |             | % Inhibition |
|----------------------------|--------------------------------------------|-------------|--------------|
|                            | – inhibitor                                | + inhibitor |              |
| 0.1                        | 10                                         | 2.5         | 75           |
| 0.5                        | 10                                         | 2.5         | 75           |
| 1.0                        | 10                                         | 3.5         | 65           |
| 2.0                        | 10                                         | 6.0         | 40           |
| 3.0                        | 10                                         | 8.0         | 20           |
| 4.0                        | 10                                         | 10.0        | 0            |

without inhibitor had an average specific activity of 7500 nmol/min/mg of protein, whereas when the enzyme incubated with untrypsinized inhibitor had specific activity, 1900 nmol/min/mg protein. Full enzyme activity was obtained after the inhibitor had been incubated with trypsin for 45 min, however 50% of inhibition was reversed after the treatment of inhibitor with trypsin for 15 min. These data are a further indication that the inhibitor is a protein.

#### *Effect of different concentrations of bovine serum albumin on inhibitor activity*

The above results suggest that the inhibitor is not exerted its effect by covalent phosphorylation or by allosteric modulation. The experiment was designed to investigate the effects of high concentrations of serum albumin on inhibitor activity. Table II revealed that when the concentration of albumin increased to 4 mg/ml, full enzyme activity was obtained. This inhibitor is in contrast to other protein inhibitor which reported earlier [8] where the activity of the later did not alter at high concentration of albumin (unpublished data).

## Discussion

Short-term regulation of fatty acid synthesis was first demonstrated with liver slices where insulin increased the incorporation of [ $^{14}\text{C}$ ]acetate into fatty acids [18], however, glucagon and cyclic AMP de-

creases its synthesis [19]. Lactate was reported to increase both fatty acid synthesis and acetyl CoA carboxylase activity [20]. In the last 15 years several mechanisms have been reported on the regulation of acetyl CoA carboxylase activity. Short-term regulation of the carboxylase may be subject to control by allosteric effectors, its state of aggregation, covalent modification and/or the presence of regulatory proteins.

Several reports from Lane's laboratory [21–23] suggested that the changes in protomer equilibrium and hence the activity of acetyl CoA carboxylase and fatty acid synthesis *in vivo* are demonstrated by the cytosolic concentration of citrate. Furthermore the dramatic decrease in fatty acid synthesis caused by dibutyryl cyclic AMP and glucagon was accompanied by depolymerization of the enzyme. On the other hand, it is reported that the inhibition of fatty acid synthesis by glucagon *in vivo* did not control by the decrease in the citrate level [24]. Our previous report [10] indicated that acetyl CoA carboxylase *in vitro* was maximum at the physiological concentration of citrate (0.5 mM), however a gradual increase in citrate concentration (up to 28 mM) resulted in a gradual decrease in enzyme activity. Also the magnitude of enzyme inhibition by the protein inhibitor, under these citrate concentrations, remained unchanged [10]. In the present study, we also demonstrated that the large molecular weight protein inhibitor is not producing its inhibitory effect by depleting citrate from the incubation mixture. Hence, evidence from our studies [8–11] and others [24] suggest that citrate may not play a direct role in regulating acetyl CoA carboxylase activity or fatty acid synthesis.

It was first reported by Carlson and Kim (25) that acetyl CoA carboxylase activity is regulated via a phosphorylation (inactive) and dephosphorylation (active enzyme) mechanism. Inactivation of the carboxylase through phosphorylation is catalyzed by cyclic AMP-dependent and independent protein kinases [3, 17, 26]. Several reports argued against the phosphorylation mechanism based on the following reasons: (a) Highly purified and active acetyl CoA carboxylase contained bound phosphate [27]. (b) Incorporation of  $^{32}\text{P}_i$  into carboxylase did not affect enzyme activity [28–30]. (c) Acetyl CoA carboxylase was inactivated by protein inhibitor without the involvement of phosphorylation [8–11]. Furthermore, a very recent report by Witters [31] indicated that the



effect of both insulin and glucagon on phosphorylation and acetyl CoA carboxylase inhibition is additive. In contrast to these findings, Porter [32] reported that insulin could abolish the inactivation of acetyl CoA carboxylase produced by glucagon. Despite the major differences in these two studies [31, 32], it is surprising that both laboratories are in agreement on the covalent phosphorylation mechanism. Moreover, despite earlier reports [25, 33] indicating that acetyl CoA carboxylase is regulated by a phosphorylation/dephosphorylation cycle only, later reports [4, 26] from these laboratories have shifted to a new approach, that regulation of carboxylase activity may be a consequence of both allosteric modulation and covalent enzyme phosphorylation. Hence, it is apparent that the exact mechanism(s) of regulation of the carboxylase is not yet resolved.

The present study, as well as our previous studies [8–11], reveal that acetyl CoA carboxylase is not regulated by either covalent phosphorylation, its state of aggregation or by allosteric modulation, however, it appears that two cytosolic proteins may

play the actual role in regulating acetyl CoA carboxylase activity and consequently fatty acid synthesis. These two endogenous proteins differ from each other, that the reported protein inhibitor in this communication has a large molecular weight and its inhibiting activity can be reversed by increasing bovine serum albumin concentration in the incubating mixture.

Preliminary experiments have also been carried out to determine the variation of the Protein inhibitor in livers of rats subjected to different nutritional and hormonal states. The maximum amount of inhibitor was found in animals fasted for 48 h and the minimum amount was demonstrated in livers of animals fasted 48 h and refed fat free diet for 48 h.

#### Acknowledgments

Supported in part by grants AM01388, AM21148 from National Institute of Health. The authors are grateful to Dr. J. W. Porter for his support. We thank Ms Elisa Pintar for preparing the manuscript.

- [1] S. Numa, T. Gotto, E. Ringelmann, and P. Riedel, *Biochem. Biophys. Acta* **84**, 750 (1967).
- [2] K.-H. Kim, *Mol. Cell Biochem.* **28**, 27 (1979).
- [3] D. G. Hardie and P. Cohen, *FEBS Lett.* **103**, 333 (1979).
- [4] L. A. Witters, E. M. Kowaloff, and J. Avruch, *J. Biol. Chem.* **254**, 245 (1979).
- [5] M.-S. Shaio, R. Drong, and J. Porter, *Biochem. Biophys. Res. Commun.* **98**, 80 (1981).
- [6] R. Brownsey, G. J. Belsham, and R. M. Denton, *FEBS Lett.* **124**, 145 (1981).
- [7] K. Bloch and D. Vance, *Ann. Rev. Biochem.* **46**, 263 (1977).
- [8] M. N. Abdel-Halim and J. W. Porter, *J. Biol. Chem.* **255**, 441 (1980).
- [9] M. N. Abdel-Halim and S. Yousufzai, *Int. J. Biochem.* **13**, 1171 (1981).
- [10] M. N. Abdel-Halim and S. Yousufzai, *Int. J. Biochem.* In press.
- [11] M. N. Abdel-Halim and S. Yousufzai, *Experientia*, **37**, 1167 (1981).
- [12] P. W. Marjerus, R. Jacobs, M. Smith, and H. Smith, *J. Biol. Chem.* **243**, 3588 (1968).
- [13] L. Ornstein and B. Davis, *Ann. N. Y. Acad. Sci.* **121**, 404 (1964).
- [14] U. K. Laemmli, *Nature* **227**, 680 (1970).
- [15] H. O. Lowry, N. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
- [16] K.-H. Lee and K.-H. Kim, *J. Biol. Chem.* **252**, 1748 (1977).
- [17] D. J. Hardie and P. Cohen, *FEBS Lett.* **91**, (1978).
- [18] K. Bloch and W. Kramer, *J. Biol. Chem.* **173**, 811 (1948).
- [19] M. Akhtar and D. P. Bloxham, *Biochem. J.* **120**, 11p (1970).
- [20] M. Geelen, R. Harres, A. Beynen, and S. McCune, *Diabetes* **29**, 1006 (1980).
- [21] M. D. Lane, J. Moss, and S. E. Polakis, *Curr. Top. Cell. Regul.* **8**, 139 (1974).
- [22] P. A. Watkins, D. M. Torlow, and M. D. Lane, *Proc. Nat. Acad. Sci. (USA)* **74**, 1497 (1977).
- [23] M. D. Lane, P. A. Watkins, and M. J. Meredith, *CRC. Crit. Rev. Biochem.* **7**, 121 (1979).
- [24] G. A. Cook, R. Nielsen, R. Hawkins, M. Mehlman, M. Lakshman, and R. Veech, *J. Biol. Chem.* **252**, 4421 (1977).
- [25] C. A. Carlson and K.-H. Kim, *J. Biol. Chem.* **248**, 378 (1977).
- [26] B. A. Lent, K.-H. Lee, and K.-H. Kim, *J. Biol. Chem.* **253**, 8149 (1978).
- [27] H. Inoue and J. M. Lowenstein, *J. Biol. Chem.* **247**, 4825 (1972).
- [28] R. W. Brownsey, W. Hughes, R. Denton, and R. Meyer, *Biochem. J.* **168**, 441 (1977).
- [29] P. H. Pekala, M. J. Meredith, D. M. Tarlow, and M. D. Lane, *J. Biol. Chem.* **253**, 5267 (1978).
- [30] P. R. Desjardins and K. Dakshinamurti, *Int. J. Biochem.* **9**, 227 (1978).
- [31] L. A. Witters, *Biochem. Biophys. Res. Commun.* **100**, 871 (1981).
- [32] J. W. Porter, *FEBS (D. Raymond and J. Knudsen, eds.)* **46**, 41 (1978).
- [33] J. Avruch, L. A. Witters, M. C. Alexander, and M. A. Bush, *J. Biol. Chem.* **253**, 1511 (1978).